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METHOD FOR DETECTING OVARIAN CANCER

Abstract:

Abstract of WO0235232

A method for the diagnosis, prognosis, and monitoring of ovarian cancer in a subject by detecting hK6 in a sample from the subject, preferably a serum sample or tumor tissue extract. hK6 may be measured using a reagent that detects or binds to hK6 preferably antibodies specifically reactive with hK6 or a part thereof. Imaging methods for tumors associated with hK6 are also described using an agent that binds to hK6 which has a label for imaging the tumor.

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(71) Applicant *(for all designated States except US)*: **MOUNT SINAI HOSPITAL** [CA/CA]; 600 University Avenue, Toronto, Ontario M5G 1X5 (CA).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **DIAMANDIS, Eleftherios, P.** [CA/CA]; 1504 Gerrard Street West, Suite 44, Toronto, Ontario M5G 2X2 (CA).

(74) Agent: **BERESKIN & PARR**; 40 King Street West, Box 401, 40th Floor, Toronto, Ontario M5H 3Y2 (CA).

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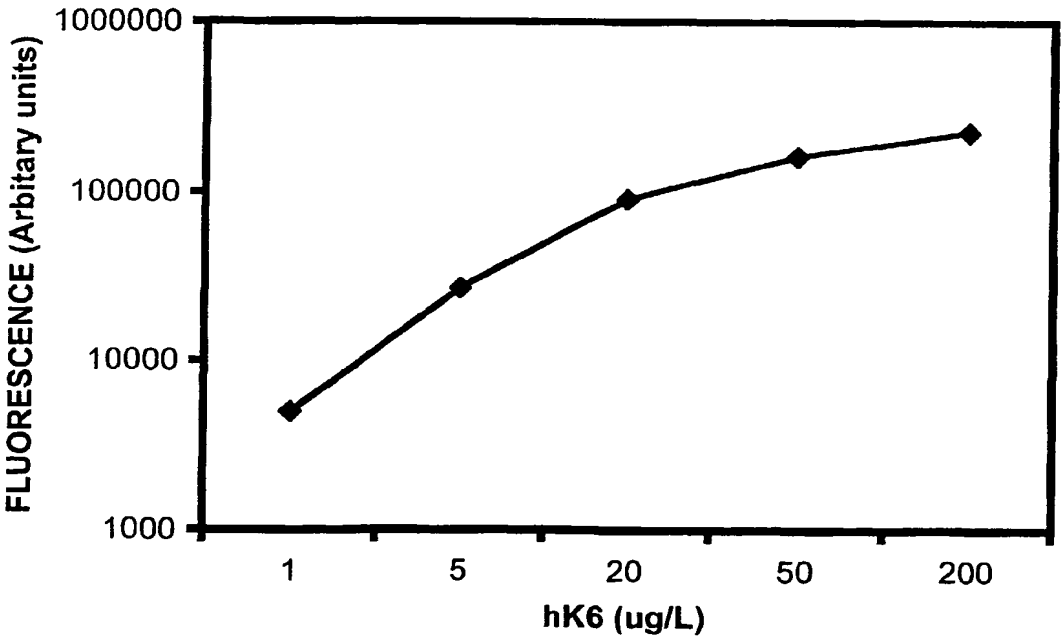

(57) Abstract: A method for the diagnosis, prognosis, and monitoring of ovarian cancer in a subject by detecting hK6 in a sample from the subject, preferably a serum sample or tumor tissue extract. hK6 may be measured using a reagent that detects or binds to hK6 preferably antibodies specifically reactive with hK6 or a part thereof. Imaging methods for tumors associated with hK6 are also described using an agent that binds to hK6 which has a label for imaging the tumor.

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TITLE: Methods for Detecting Ovarian Cancer

FIELD OF THE INVENTION

The invention relates to diagnostic and prognostic methods for ovarian carcinoma.

BACKGROUND OF THE INVENTION

Until recently, the human kallikrein gene family was thought to consist of only 3 genes: pancreatic/renal kallikrein (KLK1, encoding for hK1 protein), human glandular kallikrein 2 (KLK2, encoding for hK2 protein) and human kallikrein 3 (KLK3, encoding for hK3 protein or prostate-specific antigen, PSA). The latter two kallikreins, PSA and hK2, are relatively prostatic-specific and they have already found important applications as biomarkers for the diagnosis and monitoring of prostate cancer (1-6).

New members of the human kallikrein gene family have recently been discovered (1). This gene family now contains at least 14 genes which are all encoding for serine proteases, show significant homology at both the DNA and amino acid level and they are all localized at the chromosomal locus 19q13.3-q13.4, in tandem, without any intervention from other non-kallikrein genes. This area of investigation has recently been reviewed (1).

The KLK6 gene (encoding for human kallikrein 6, hK6) has been cloned independently by three groups of investigators and was previously given the names zyme (7), protease M (8) and neurosin (9). Recently, uniform nomenclature for all newly discovered and the traditional kallikrein genes has been established (10). The KLK6 gene encodes for a trypsin-like serine protease of 244 amino acids in length, of which 16 amino acids constitute the signal peptide and 5 amino acids, the activation peptide. The mature enzyme consists of 223 amino acids. It has been previously predicted that hK6 is a secreted protein (7-9,11). This was recently verified by finding hK6 protein in various biological fluids, including cerebrospinal fluid, nipple aspirate fluid, breast cyst fluid, male and female serum, seminal plasma, amniotic fluid and breast cancer cytosols (12). Little et al. (7) have demonstrated that this enzyme has amyloidogenic potential in the brain and may play a role in the development and progression of Alzheimer's disease. Others have cloned the same gene by the method of differential display, and found that it is down-regulated in aggressive forms of breast cancer (8). The same gene was cloned by Yamashiro et al. from the human colon adenocarcinoma cell line COLO 201 (9).

Among the classical human kallikreins, PSA has proven to be the most valuable biomarker for prostate cancer and is currently used for diagnosis and monitoring of this disease (2-4). Another potential prostatic biomarker, hK2, has also been recently introduced (5, 6). Among the newly discovered kallikreins (1), none of them has been examined as a serological marker for any malignancy since no methods currently exist to measure the secreted proteins with high sensitivity and specificity.

Ovarian cancer is a serious disease which causes more deaths than any other cancer of the female reproductive system (13). Since survival could be dramatically improved if the disease is diagnosed early (14), there is great interest in the identification of biomarkers that could aid in the early detection and facilitate grading and/or staging (15). Unfortunately, the current serological markers for ovarian carcinoma,

including CA125 (16-19), inhibin (20-23), OVX1 (24) as well as many other markers (reviewed in 25) have shown some promise but have not gained wide clinical acceptance. Another potential ovarian cancer marker, lysophosphatidic acid appears to also have some value for this purpose (26).

There is an urgent need for discovery and validation of new biomarkers for ovarian carcinoma. Early diagnosis of ovarian cancer, particularly with serological analysis, may improve clinical outcomes through administration of effective treatment.

SUMMARY OF THE INVENTION

A highly sensitive hK6 immunoassay for the measurement of hK6 in various biological fluids was developed (Example 1). Using this sensitive assay the hK6 concentration in serum was found to be significantly increased in a large proportion of patients with ovarian cancer. In particular, hK6 was found to be significantly increased in ovarian cancer patients when compared to normal non-cancer patients and patients with benign disease. Thus, hK6 constitutes a new biomarker for diagnosis and monitoring of ovarian cancer. hK6 may be used to diagnose and monitor late stage ovarian cancer, and it may be used as a biomarker before surgery or after relapse.

The present inventors also quantitated the amount of hK6 in extracts of ovarian tumors and determined that the amount of hK6 correlated with clinicopathological variables documented at the time of surgical excision and with progression free survival and overall survival. Increased hK6 levels were found to be predictive of more aggressive tumor behavior over time. hK6 positivity was found to be associated with about a 2-fold increase in the risk of both disease progression and of death.

hK6, and agents that bind to hK6 may be used to detect ovarian cancer and in particular they can be used in the diagnostic evaluation of ovarian cancer, and the identification of subjects with a predisposition to ovarian cancer.

The present invention relates to a method for diagnosing and monitoring ovarian cancer in a subject comprising measuring hK6 in a sample from the subject. hK6 may be measured using a reagent that detects or binds to hK6 preferably antibodies specifically reactive with hK6 or a part thereof.

In an aspect of the invention, a method is provided for detecting hK6 associated with ovarian cancer in a patient comprising:

(a) taking a sample derived from a patient;

(b) detecting or identifying in the sample hK6; and

(c) comparing the detected amount with an amount detected for a standard.

The invention also relates to a method of screening a subject for ovarian cancer comprising: (a) obtaining a biological sample from a subject; (b) detecting the amount of hK6 in said sample; and (c) comparing said amount of hK6 detected to a predetermined standard, where detection of a level of hK6 greater than that of a standard indicates the presence of ovarian cancer, in particular late stage ovarian cancer.

The terms "detecting" or "detect" include assaying, quantitating, imaging or otherwise establishing the presence or absence of the target hK6, subunits thereof, or combinations of reagent bound targets, and the like, or assaying for, imaging, ascertaining, establishing, or otherwise determining one or more factual

characteristics of ovarian cancer, metastasis, stage, or similar conditions. The term encompasses diagnostic, prognostic, and monitoring applications for hK6.

In an embodiment, the invention relates to a method for detecting ovarian cancer in a subject by quantitating hK6 in a biological sample from the subject comprising (a) reacting the biological sample with an antibody specific for hK6 which is directly or indirectly labelled with a detectable substance; and (b) detecting the detectable substance.

The invention further relates to a method for diagnosing and monitoring ovarian carcinoma in a subject by quantitating hK6 in a sample from a subject comprising (a) reacting a biological sample from the subject with an antibody specific for hK6 which is directly or indirectly labelled with a detectable substance; (b) and detecting the detectable substance.

Embodiments of the methods of the invention involve (a) reacting a biological sample from a subject with an antibody specific for hK6 which is directly or indirectly labelled with an enzyme; (b) adding a substrate for the enzyme wherein the substrate is selected so that the substrate, or a reaction product of the enzyme and substrate forms fluorescent complexes; (c) quantitating hK6 in the sample by measuring fluorescence of the fluorescent complexes; and (d) comparing the quantitated levels to that of a standard. The standard may correspond to levels obtained for other samples from the subject patient, or control subjects. In an embodiment the quantitated levels are compared to levels quantitated for subjects without ovarian carcinoma wherein an increase in hK6 levels compared with the control subjects is indicative of ovarian carcinoma, in particular late stage ovarian carcinoma.

A preferred embodiment of the invention comprises the following steps

(a) incubating a biological sample with a first antibody specific for hK6 which is directly or indirectly labeled with a detectable substance, and a second antibody specific for hK6 which is immobilized;

(b) separating the first antibody from the second antibody to provide a first antibody phase and a second antibody phase;

(c) detecting the detectable substance in the first or second antibody phase thereby quantitating hK6 in the biological sample; and

(d) comparing the quantitated hK6 with levels for a standard.

The standard may correspond to levels quantitated for samples from healthy control subjects, from subjects with benign disease, subjects with early stage disease, or from other samples of the subject. Increased levels of hK6 as compared to the standard may be indicative of ovarian cancer, in particular late stage ovarian cancer.

The invention also contemplates the methods described herein using multiple markers for ovarian cancer. Therefore, the invention contemplates a method for anaylzing a biological sample for the presence of hK6 and other markers that are specific indicators of ovarian cancer. Other markers include markers to kallikreins such as human stratum corneum chymotryptic enzyme (HSCCE), kallikrein 4, kallikrein 5, kallikrein 8, kallikrein 9, kallikrein 10, kallikrein 11; CA125, CA15-3, CA19-9, OVX1, lysophosphatidic acid

(LPA) and carcinoembryonic antigen (CEA). Preferably the other markers are markers to kallikreins. In a preferred embodiment, the markers are two or more of hK6, hK10, and CA 125. The methods described herein may be modified by including reagents to detect the additional markers, or nucleic acids for the markers.

The invention also relates to a method for imaging a tumor associated with hK6 comprising

(a) incubating the tumor with an agent that binds to hK6 for a sufficient period of time to permit the agent to bind to hK6 associated with the tumor, where the agent carries a label for imaging the tumor;

(b) detecting the presence of the label localized to the tumor.

In accordance with an aspect of the invention an in vivo method is provided comprising administering to a subject an agent that has been constructed to target one or more kallikreins.

The invention therefore contemplates an in vivo method comprising administering to a mammal one or more agent that carries a label for imaging and binds to a kallikrein, preferably hK6, and then imaging the mammal.

According to a preferred aspect of the invention, an in vivo method for imaging ovarian cancer is provided comprising:

(a) injecting a patient with an agent that binds to kallikrein 6, the agent carrying a label for imaging the ovarian cancer;

(b) allowing the agent to incubate in vivo and bind to kallikrein 6 associated with the ovarian cancer; and

(c) detecting the presence of the label localized to the ovarian cancer.

In an embodiment of the invention the agent is an antibody which recognizes the kallikrein. In another embodiment of the invention the agent is a chemical entity which recognizes the kallikrein.

The agent carries a label to image the kallikreins. Examples of labels useful for imaging are radiolabels, fluorescent labels (e.g fluorescein and rhodamine), nuclear magnetic resonance active labels, positron emitting isotopes detectable by a positron emission tomography ("PET") scanner, chemiluminescers such as luciferin, and enzymatic markers such as peroxidase or phosphatase. Short-range radiation emitters, such as isotopes detectable by short-range detector probes can also be employed

The invention also contemplates the localization or imaging methods described herein using multiple markers for ovarian cancer. For example, a method for imaging ovarian cancer may further comprise injecting the patient with one or more of an agent that binds to human stratum corneum chymotryptic enzyme (HSCCE), kallikrein 4, kallikrein 5, kallikrein 8, kallikrein 9, kallikrein 10, kallikrein 11, CA125, CA15-3, CA19-9, OVX1, lysophosphatidic acid (LPA) or carcinoembryonic antigen (CEA), preferably CA 125.

The invention also relates to kits for carrying out the methods of the invention.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since

various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 is a calibration curve for a hK6 protein assay. The fluorescence of the zero standard (~ 18,000 arbitrary fluorescence units) was subtracted from all other measurements.

Figure 2 shows the results of high performance liquid chromatography separation of three biological fluids and analysis of all fractions with the developed hK6 immunoassay. In all three fluids, a single immunoreactive peak around fractions 38 – 42 was detected, corresponding to a molecular mass of ~ 30 kDa. The column was calibrated with molecular weight standards (shown on top with arrows; masses are in kDa). The milk sample was diluted 10 times before injection into the HPLC column.

Figure 3 is a graph showing the results of the analysis of various human tissue cytosolic extracts for hK6 protein

Figure 4 is a graph showing the frequency distribution of hK6 concentrations in the serum of 80 patients with ovarian carcinoma. The level of 15 μg/L which was used as a cutoff in Table 1 is indicated by an arrow. About 66% of ovarian cancer patients have serum hK6 concentration higher than this cutoff value. From another 298 serum samples with non-ovarian cancer, only 2 sera had values slightly higher than 15 μg/L (see Table 1).

Figure 5 is a graph showing the correlation between hK6 and CA125 concentration in 96 serum samples from ovarian cancer patients.

Figure 6 are graphs showing the analysis of hK6 and CA125 in serial serum samples from patients with ovarian cancer. These data suggest that hK6 may have value for patient monitoring.

Figure 7A is a graph showing the distribution of hK6 in normal, to benign, to cancer patients.

Figure 7B is a graph showing the distribution of CA 125 in normal, to benign, to cancer patients.

Figure 8 is a graph showing the concentration of hK6 in pre-surgical and post-surgical serum samples of ovarian cancer patients.

Figure 9 is a graph showing the correlation between serum hK6 and CA125 concentrations.

Figure 10 is a graph showing the sensitivity and specificity of serum hK6 concentrations.

Figure 11A is a graph showing hK6 concentration versus stage of ovarian cancer.

Figure 11B is a graph showing hK6 concentration versus grade of ovarian cancer.

Figure 12A is a graph showing the survival probability versus progression –free survival (PFS).

Figure 12B is a graph showing the survival probability versus overall survival (OS).

Figure 13(A) is a graph showing the frequency distribution of hK6 specific activity in ovarian tumor extracts. The value of 35ng/mg of total protein corresponds to the limit that, according to Chi square analysis, gives the best prediction of overall survival of the study population. (See Figure 13(B) for Chi square plot.) Tumors with hK6 in excess of 35 ng/mg total protein were classified as hK6 positive and those with values less than or equal to 35 ng/mg total protein were classified as hK6 negative. 30% of the tumors

were classified as positive by this criterion. (B) Plot of hK6 tumor specific activity versus Chi-square statistic to determine the limit between hK6 positive and hK6 negative tumors that is most predictive of overall survival. Maximum predictive potential occurred between 28 to 40 ng hK6 total extract protein with a peak at 35 ng hK6/mg total extract protein.

Figure 14 is a graph showing a comparison of hK6 concentration in extracts from normal ovarian tissues ("normal"), and ovarian cancer ("cancer"). N indicates the number of specimens in each group. Horizontal bars represent the median hK6 specific activity (ng hK6/mg total extract protein) in each group. The Krustal Wallis test showed that extracted hK6 specific activity was significantly elevated in the ovarian tumor preparations ($P<0.001$).

Figure 15 is a graph showing the distribution of hK6 specific activity (ng hK6/mg total protein) in tumor extracts from stage I/II and stage III/IV ovarian cancer patients. N indicates the number of tumors comprising each group. Horizontal bars represent the median value of hK6 tumor specific activity. The Mann-Whitney test demonstrated that hK6 specific activity was significantly elevated in tumors from patients with stage III/IV ovarian cancer ($P=0.002$).

Figure 16 shows Kaplan-Meier survival curves of the entire patient population under study:effect of hK6 status. Top: progression-free survival (PFS). Bottom: overall survival (OS). The patient number in each group (n) is indicated as is the statistical significance (P value) of the survival difference between hK6 positive and hK6 negative groups. The adverse effect of hK6 positivity on both time to progression and overall survival was significant.

Figure 17 are graphs showing the effect of hK6 status (positive or negative) on progression –free survival (PFS) and on overall survival (OS) among patients with Grade I and II ovarian tumor. The patient number in each group (n) is indicated as is the statistical significance (P value) of the survival difference between hK6 positive and hK6 negative individuals. The adverse effect of hK6 positivity both on time to progression and on overall survival was significant ($P\leq 0.002$).

Figure 18 is a blot showing immunohistochemical localization of hK6 in ovarian neoplasms of varying malignant potential, cell type, and origin (epithelial versus mesenchymal). (A) Invasive papillary serous adenocarcinoma, the common malignant epithelial tumor of the ovary. Note strong cytoplasmic staining of many tumor cells, and absence of any staining of stroma or vessels. (B) Serous cystadenofibroma, a benign, mixed epithelial and fibrous neoplasm. Innumostaining is absent in the fibrous component, but strongly positive in the cytoplasm of the epithelium lining the cysts. (C) Ovarian leiomyoma, a benign smooth muscle tumor. Note the absence of staining. (D) Mucinous epithelial tumor of low malignant potential, an epithelial tumor of intermediate grade. Note weak, diffuse cytoplasmic staining of neoplastic epithelium and absent staining in supportive stroma (far left).

DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned, the present invention provides a method for monitoring, diagnosing, or for the prognosis of ovarian carcinoma in a subject by detecting hK6 in a biological sample from the subject. In an embodiment, the method comprises reacting the sample with an agent that binds to hK6,

preferably an antibody specific for hK6 which is directly or indirectly labelled with a detectable substance, and detecting the detectable substance.

The methods of the invention may be used for the detection of either an over- or an under-abundance of hK6 relative to a non- disorder state or the presence of a modified (e.g., less than full length) hK6 which correlates with a disorder state (e.g ovarian cancer), or a progression toward a disorder state. The methods described herein may be used to evaluate the probability of the presence of malignant or pre-malignant cells, for example, in a group of cells freshly removed from a host. Such methods can be used to detect tumors, quantitate their growth, and help in the diagnosis and prognosis of disease. The methods can be used to detect the presence of cancer metastasis, as well as confirm the absence or removal of all tumor tissue following surgery, cancer chemotherapy, and/or radiation therapy. They can further be used to monitor cancer chemotherapy and tumor reappearance.

The methods of the invention are particularly useful in the diagnosis of late stage ovarian carcinoma and for the prognosis of ovarian carcinoma disease progression and mortality. As illustrated herein increased levels of hK6 detected in serum compared to a standard are indicative of late stage disease, and increased levels of hK6 in tumor tissues or extracts thereof compared to a standard are indicative of increased risk of disease progression and mortality.

The terms "sample", "biological sample", and the like mean a material known to or suspected of expressing or containing hK6. The test sample can be used directly as obtained from the source or following a pretreatment to modify the character of the sample. The sample can be derived from any biological source, such as tissues or extracts, including cells (e.g. tumor cells) and physiological fluids, such as, for example, whole blood, plasma, serum, saliva, ocular lens fluid, cerebral spinal fluid, sweat, urine, milk, ascites fluid, synovial fluid, peritoneal fluid and the like. The sample can be obtained from animals, preferably mammals, most preferably humans. The sample can be treated prior to use, such as preparing plasma from blood, diluting viscous fluids, and the like. Methods of treatment can involve filtration, distillation, extraction, concentration, inactivation of interfering components, the addition of reagents, and the like. Proteins may be isolated from the samples and utilized in the methods of the invention. In a preferred embodiment, the biological sample is serum or tumor tissue extracts, most preferably serum.

In embodiments of the invention, the method described herein is adapted for diagnosing and monitoring, and for the prognosis of ovarian carcinoma by detecting hK6 in biological samples from a subject. These applications require that the amount of hK6 detected in a sample from a subject being tested be compared to levels detected for another sample or an earlier sample from the subject, or levels detected for a control sample. Levels for control samples from healthy subjects or subjects with benign disease may be established by prospective and/or retrospective statistical studies. Healthy subjects who have no clinically evident disease or abnormalities may be selected for statistical studies. Diagnosis may be made by a finding of statistically different levels of hK6 compared to a control sample or previous levels detected for the same subject.

The term "hK6" refers to human kallikrein 6, (also known as zyme, protease M, and neurosin) a

trypsin-like serine protease of 244 amino acids in length, of which 16 amino acids constitute the signal peptide and 5 amino acids, the activation peptide (7, 8, and 9). The term includes all homologs, naturally occurring allelic variants, isoforms and precursors of human kallikrein 6 of GenBank Accession Nos. AF013988, AF149289, HSU62801, D78203, and NM002774. In general for example, naturally occurring allelic variants of human kallikrein 6 will share significant homology (70-90%) to the sequences shown in GenBank Accession Nos. AF013988, AF149289, HSU62801, D78203, and NM002774. Allelic variants may contain conservative amino acid substitutions from the KLK6 sequence or will contain a substitution of an amino acid from a corresponding position in a hK6 homologue such as, for example, the murine kallikrien 6 homologue.

The term "subject" refers to a warm-blooded animal such as a mammal which is afflicted with or suspected to be afflicted with ovarian cancer. Preferably, "subject" refers to a human.

The antibodies specific for hK6 used in the methods of the invention may be obtained from scientific or commercial sources. Alternatively, isolated native hK6 or recombinant hK6 may be utilized to prepare antibodies, monoclonal or polyclonal antibodies, and immunologically active fragments (e.g. a Fab or $(Fab)_2$ fragment), an antibody heavy chain, an antibody light chain, humanized antibodies, a genetically engineered single chain F_v molecule (Ladner et al, U.S. Pat. No. 4,946,778), or a chimeric antibody, for example, an antibody which contains the binding specificity of a murine antibody, but in which the remaining portions are of human origin. Antibodies including monoclonal and polyclonal antibodies, fragments and chimeras, may be prepared using methods known to those skilled in the art. Preferably, antibodies used in the methods of the invention are reactive against hK6 if they bind with a K_a of greater than or equal to 10^{-7} M. In a sandwich immunoassay of the invention mouse polyclonal antibodies and rabbit polyclonal antibodies are utilized.

Antibodies specifically reactive with hK6, or derivatives, such as enzyme conjugates or labeled derivatives, may be used to detect hK6 in various biological samples, for example they may be used in any known immunoassays which rely on the binding interaction between an antigenic determinant of a protein and the antibodies. Examples of such assays are radioimmunoassays, enzyme immunoassays (e.g.ELISA), immunofluorescence, immunoprecipitation, latex agglutination, hemagglutination, and histochemical tests.

An antibody specific for hK6 may be labelled with a detectable substance and localised or identified in biological samples based upon the presence of the detectable substance. Examples of detectable substances include, but are not limited to, the following: radioisotopes (e.g., ^{3}H, ^{14}C, ^{35}S, ^{125}I, ^{131}I), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), luminescent labels such as luminol; enzymatic labels (e.g., horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase, acetylcholinesterase), biotinyl groups (which can be detected by marked avidin e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or calorimetric methods), predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). Indirect methods may also be employed in which the primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity

for the antibody reactive against hK6. By way of example, if the antibody having specificity against hK6 is a rabbit IgG antibody, the second antibody may be goat anti-rabbit gamma-globulin labelled with a detectable substance as described herein.

Methods for conjugating or labelling the antibodies discussed above may be readily accomplished by one of ordinary skill in the art. (See for example Inman, Methods In Enzymology, Vol. 34, Affinity Techniques, Enzyme Purification: Part B, Jakoby and Wichek (eds.), Academic Press, New York, p. 30, 1974; and Wilchek and Bayer, "The Avidin-Biotin Complex in Bioanalytical Applications,"Anal. Biochem. 171:1-32, 1988 re methods for conjugating or labelling the antibodies with enzyme or ligand binding partner).

Time-resolved fluorometry may be used to detect a signal. For example, the method described in Christopoulos TK and Diamandis EP Anal Chem 1992:64:342-346 may be used with a conventional time-resolved fluorometer.

Therefore, in accordance with an embodiment of the invention, a method is provided wherein a hK6 antibody is labelled with an enzyme, a substrate for the enzyme is added wherein the substrate is selected so that the substrate, or a reaction product of the enzyme and substrate, forms fluorescent complexes with a lanthanide metal. A lanthanide metal is added and hK6 is quantitated in the sample by measuring fluorescence of the fluorescent complexes. The antibodies specific for hK6 may be directly or indirectly labelled with an enzyme. Enzymes are selected based on the ability of a substrate of the enzyme, or a reaction product of the enzyme and substrate, to complex with lanthanide metals such as europium and terbium. Examples of suitable enzymes include alkaline phosphatase and β-galactosidase. Preferably, the enzyme is alkaline phosphatase. The hK6 antibodies may also be indirectly labelled with an enzyme. For example, the antibodies may be conjugated to one partner of a ligand binding pair, and the enzyme may be coupled to the other partner of the ligand binding pair. Representative examples include avidin-biotin, and riboflavin-riboflavin binding protein. Preferably the antibodies are biotinylated, and the enzyme is coupled to streptavidin.

In the method, antibody bound to hK6 in a sample is detected by adding a substrate for the enzyme. The substrate is selected so that in the presence of a lanthanide metal (e.g. europium, terbium, samarium, and dysprosium, preferably europium and terbium), the substrate, or a reaction product of the enzyme and substrate, forms a fluorescent complex with the lanthanide metal. Examples of enzymes and substrates for enzymes that provide such fluorescent complexes are described in U.S. Patent No. 5,3112,922 to Diamandis. By way of example, when the antibody is directly or indirectly labelled with alkaline phosphatase the substrate employed in the method may be 4-methylumbelliferyl phosphate, or 5-fluorosalicyl phosphate. The fluorescence intensity of the complexes is typically measured using a time-resolved fluorometer e.g. a CyberFluor 615 Imunoanalyzer (Nordion International, Kanata, Ontario).

The sample, an antibody specific for hK6, or hK6 may be immobilized. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose, liposomes, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, well, beads, disc, sphere etc. The immobilized

antibody may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

In accordance with an embodiment, the present invention provides means for determining hK6 in a blood sample or tumor tissue extract, preferably a serum sample, by measuring hK6 by immunoassay. It will be evident to a skilled artisan that a variety of immunoassay methods can be used to measure hK6. In general, an hK6 immunoassay method may be competitive or noncompetitive. Competitive methods typically employ an immobilized or immobilizable antibody to hK6 (anti-hK6) and a labeled form of hK6. Sample hK6 and labeled hK6 compete for binding to anti-hK6. After separation of the resulting labeled hK6 that has become bound to anti-hK6 (bound fraction) from that which has remained unbound (unbound fraction), the amount of the label in either bound or unbound fraction is measured and may be correlated with the amount of hK6 in the test sample in any conventional manner, e.g., by comparison to a standard curve.

Preferably a non-competitive method is used for the determination of hK6, with the most common method being the "sandwich" method. In this assay, two anti-hK6 antibodies are employed. One of the anti-hK6 antibodies is directly or indirectly labeled (sometimes referred to as the "detection antibody") and the other is immobilized or immobilizable (sometimes referred to as the "capture antibody"). The capture and detection antibodies can be contacted simultaneously or sequentially with the test sample. Sequential methods can be accomplished by incubating the capture antibody with the sample, and adding the detection antibody at a predetermined time thereafter (sometimes referred to as the "forward" method); or the detection antibody can be incubated with the sample first and then the capture antibody added (sometimes referred to as the "reverse" method). After the necessary incubation(s) have occurred, to complete the assay, the capture antibody is separated from the liquid test mixture, and the label is measured in at least a portion of the separated capture antibody phase or the remainder of the liquid test mixture. Generally it is measured in the capture antibody phase since it comprises hK6 bound by ("sandwiched" between) the capture and detection antibodies.

In a typical two-site immunometric assay for hK6, one or both of the capture and detection antibodies are polyclonal antibodies. The label used in the detection antibody can be selected from any of those known conventionally in the art. The label may be an enzyme or a chemiluminescent moiety, but it can also be a radioactive isotope, a fluorophor, a detectable ligand (e.g., detectable by a secondary binding by a labeled binding partner for the ligand), and the like. Preferably the antibody is labelled with an enzyme which is detected by adding a substrate that is selected so that a reaction product of the enzyme and substrate forms fluorescent complexes. The capture antibody is selected so that it provides a means for being separated from the remainder of the test mixture. Accordingly, the capture antibody can be introduced to the assay in an already immobilized or insoluble form, or can be in a immobilizable form, that is, a form which enables immobilization to be accomplished subsequent to introduction of the capture antibody to the assay. An immobilized capture antibody may comprise an antibody covalently or noncovalently attached to a solid phase such as a magnetic particle, a latex particle, a microtiter plate well, a bead, a cuvette, or other reaction vessel. An example of an immobilizable capture antibody is antibody which has been chemically modified

with a ligand moiety, e.g., a hapten, biotin, or the like, and which can be subsequently immobilized by contact with an immobilized form of a binding partner for the ligand, e.g., an antibody, avidin, or the like. In an embodiment, the capture antibody may be immobilized using a species specific antibody for the capture antibody that is bound to the solid phase.

A particular sandwich immunoassay method of the invention employs two antibodies reactive against hK6, a second antibody having specificity against an antibody reactive against hK6 labelled with an enzymatic label, and a fluorogenic substrate for the enzyme. In an embodiment, the enzyme is alkaline phosphatase (ALP) and the substrate is 5-fluorosalicyl phosphate. ALP cleaves phosphate out of the fluorogenic substrate, 5-fluorosalicyl phosphate, to produce 5-fluorosalicylic acid (FSA). 5-Fluorosalicylic acid can then form a highly fluorescent ternary complex of the form FSA-Tb(3+)-EDTA, which can be quantified by measuring the Tb3+ fluorescence in a time-resolved mode. Fluorescence intensity is measured using a time-resolved fluorometer as described herein.

The above-described immunoassay methods and formats are intended to be exemplary and are not limiting since, in general, it will be understood that any immunoassay method or format can be used in the present invention.

The methods of the invention can be carried out using a diagnostic kit for quantitating hK6 in a sample. By way of example, the kit may contain antibodies specific for hK6, antibodies against the antibodies labelled with an enzyme; and a substrate for the enzyme. The kit may also contain microtiter plate wells, standards, assay diluent, wash buffer, adhesive plate covers, and/or instructions for carrying out a method of the invention using the kit.

Antibodies specific for hK6 may also be used in imaging methodologies in the management of ovarian cancer. The invention provides a method for imaging tumors associated with hK6 and optionally one or more other kallikreins, preferably kallikreins associated with ovarian cancer, including but not limited to hK4, hK5, hK8, hK9, hK10 and hK11.

The invention also contemplates imaging methods described herein using multiple markers for ovarian cancer. For example, a method for imaging ovarian cancer may utilize an agent that binds to hK6 and one or more of an agent that binds to human stratum corneum chymotryptic enzyme (HSCCE), kallikrein 4, kallikrein 5, kallikrein 8, kallikrein 9, kallikrein 10, kallikrein 11, CA125, CA15-3, CA19-9, OVX1, lysophosphatidic acid (LPA) or carcinoembryonic antigen (CEA), preferably Ca 125. Preferably each agent is labeled so that it can be distinguished during the imaging.

In an embodiment the method is an in vivo method and a subject or patient is administered one or more agents that carry an imaging label and that are capable of targeting or binding to a kallikrein, preferably hK6. The agent is allowed to incubate in vivo and bind to the kallikrein(s) associated with a tumor, preferably ovarian tumors. The presence of the label is localized to the ovarian cancer, and the localized label is detected using imaging devices known to those skilled in the art.

The agent may be an antibody or chemical entity which recognizes the kallikrein(s). In an aspect of the invention the agent is a polyclonal antibody or monoclonal antibody, or fragments thereof, or

constructs thereof including but not limited to, single chain antibodies, bifunctional antibodies, molecular recognition units, and peptides or entities that mimic peptides. The antibodies specific for the kallikreins used in the methods of the invention may be obtained from scientific or commercial sources, or isolated native kallikrein or recombinant kallikrein may be utilized to prepare antibodies etc as described herein.

An agent may be a peptide that mimics the epitope for an antibody specific for a kallikrein and binds to the kallikrein. The peptide may be produced on a commercial synthesizer using conventional solid phase chemistry. By way of example, a peptide may be prepared that includes either tyrosine lysine, or phenylalanine to which N_2S_2 chelate is complexed (See U.S. Patent No. 4,897,255). The anti-kallikrein peptide conjugate is then combined with a radiolabel (e.g. sodium ^{99m}Tc pertechnetate or sodium ^{188}Re perrhenate) and it may be used to locate a kallilkrein producing tumor.

The agent carries a label to image the kallikreins. The agent may be labelled for use in radionuclide imaging. In particular, the agent may be directly or indirectly labelled with a radioisotope. Examples of radioisotopes that may be used in the present invention are the following: ^{277}Ac, ^{211}At, ^{128}Ba, ^{131}Ba, ^{7}Be, ^{204}Bi, ^{205}Bi, ^{206}Bi, ^{76}Br, ^{77}Br, ^{82}Br, ^{109}Cd, ^{47}Ca, ^{11}C, ^{14}C, ^{36}Cl, ^{48}Cr, ^{51}Cr, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{165}Dy, ^{155}Eu, ^{18}F, ^{153}Gd, ^{66}Ga, ^{67}Ga, ^{68}Ga, ^{72}Ga, ^{198}Au, ^{3}H, ^{166}Ho, ^{111}In, ^{113m}In, ^{115m}In, ^{123}I, ^{125}I, ^{131}I, ^{189}Ir, ^{191m}Ir, ^{192}Ir, ^{194}Ir, ^{52}Fe, ^{55}Fe, ^{59}Fe, ^{177}Lu, ^{15}O, $^{191m-191}$Os, ^{109}Pd, ^{32}P, ^{33}P, ^{42}K, ^{226}Ra, ^{186}Re, ^{188}Re, ^{82m}Rb, ^{153}Sm, ^{46}Sc, ^{47}Sc, ^{72}Se, ^{75}Se, ^{105}Ag, ^{22}Na, ^{24}Na, ^{89}Sr, ^{35}S, ^{38}S, ^{177}Ta, ^{96}Tc, ^{99m}Tc, ^{201}Tl, ^{202}Tl, ^{113}Sn, ^{117m}Sn, ^{121}Sn, ^{166}Yb, ^{169}Yb, ^{175}Yb, ^{88}Y, ^{90}Y, ^{62}Zn and ^{65}Zn. Preferably the radioisotope is ^{131}I, ^{125}I, ^{123}I, ^{111}I, ^{99m}Tc, ^{90}Y, ^{186}Re, ^{188}Re, ^{32}P, ^{153}Sm, ^{67}Ga, ^{201}Tl ^{77}Br, or ^{18}F, and is imaged with a photoscanning device.

Procedures for labeling biological agents with the radioactive isotopes are generally known in the art. U.S. Pat. No. 4,302,438 describes tritium labeling procedures. Procedures for iodinating, tritium labeling, and ^{35}S labeling especially adapted for murine monoclonal antibodies are described by Goding, J. W. (supra, pp 124-126) and the references cited therein. Other procedures for iodinating biological agents, such as antibodies, binding portions thereof, probes, or ligands, are described in the scientific literature (see Hunter and Greenwood, Nature 144:945 (1962), David et al., Biochemistry 13:1014-1021 (1974), and U.S. Pat. Nos. 3,867,517 and 4,376,110). Iodinating procedures for agents are described by Greenwood, F. et al., Biochem. J. 89:114-123 (1963); Marchalonis, J., Biochem. J. 113:299-305 (1969); and Morrison, M. et al., Immunochemistry, 289-297 (1971). ^{99m} Tc-labeling procedures are described by Rhodes, B. et al. in Burchiel, S. et al. (eds.), Tumor Imaging: The Radioimmunochemical Detection of Cancer, New York: Masson 111-123 (1982) and the references cited therein. Labelling of antibodies or fragments with technetium-99m are also described for example in U.S. Pat. No. 5,317,091, U.S. Pat. No. 4,478,815, U.S. Pat. No. 4,478,818, U.S. Pat. No. 4,472,371, U.S. Pat. No. Re 32,417, and U.S. Pat. No. 4,311,688. Procedures suitable for ^{111} In-labeling biological agents are described by Hnatowich, D. J. et al., J. Immul. Methods, 65:147-157 (1983), Hnatowich, D. et al., J. Applied Radiation, 35:554-557 (1984), and Buckley, R. G. et al., F.E.B.S. 166:202-204 (1984).

An agent may also be labeled with a paramagnetic isotope for purposes of an in vivo method of the invention. Examples of elements that are useful in magnetic resonance imaging include gadolinium, terbium,

tin, iron, or isotopes thereof. (See, for example, Schaefer et al., (1989) JACC 14, 472-480; Shreve et al., (1986) Magn. Reson. Med. 3, 336-340; Wolf, G L., (1984) Physiol. Chem. Phys. Med. NMR 16, 93-95; Wesbey et al., (1984) Physiol. Chem. Phys. Med. NMR 16, 145-155; Runge et al., (1984) Invest. Radiol. 19, 408-415 for discussions on in vivo nuclear magnetic resonance imaging.)

In the case of a radiolabeled agent, the agent may be administered to the patient, it is localized to the tumor having a kallikrein with which the agent binds, and is detected or "imaged" in vivo using known techniques such as radionuclear scanning using e.g., a gamma camera or emission tomography. [See for example A. R. Bradwell et al., "Developments in Antibody Imaging", Monoclonal Antibodies for Cancer Detection and Therapy, R. W. Baldwin et al., (eds.), pp. 65-85 (Academic Press 1985)]. A positron emission transaxial tomography scanner, such as the scanner designated Pet VI located at Brookhaven National Laboratory, can also be used where the radiolabel emits positrons (e.g., ^{11}C, ^{18}F, ^{15}O, and ^{13}N).

Whole body imaging techniques using radioisotope labeled agents can be used for locating both primary tumors and tumors which have metastasized. Antibodies specific for kallikreins, or fragments thereof having the same epitope specificity, are bound to a suitable radioisotope, or a combination thereof, and administered parenterally. For ovarian cancer, administration preferably is intravenous. The bio-distribution of the label can be monitored by scintigraphy, and accumulations of the label are related to the presence of ovarian cancer cells. Whole body imaging techniques are described in U.S. Pat. Nos. 4,036,945 and 4,311,688. Other examples of agents useful for diagnosis and therapeutic use which can be coupled to antibodies and antibody fragments include metallothionein and fragments (see, U.S. Pat. No. 4,732,864). These agents are useful in diagnosis staging and visualization of cancer, in particular ovarian cancer, so that surgical and/or radiation treatment protocols can be used more efficiently.

The invention also contemplates kits for carrying out the methods of the invention. The kits include an antibody or an antibody fragment which binds specifically to an epitope of a kallikrein, and means for detecting binding of the antibody to its epitope associated with tumor cells, either as concentrates (including lyophilized compositions), which may be further diluted prior to use or at the concentration of use, where the vials may include one or more dosages. Where the kits are intended for in vivo use, single dosages may be provided in sterilized containers, having the desired amount and concentration of agents. Containers that provide a formulation for direct use, usually do not require other reagents, as for example, where the kit contains a radiolabelled antibody preparation for in vivo imaging.

The following non-limiting examples are illustrative of the present invention:

Example 1

Immunofluorometric Assay of Human Kallikrein 6 (Zyme/Protease M/Neurosin)Materials and Methods

Diflunisal phosphate (DFP) was synthesized in the laboratory (diflunisal, obtained from Sigma Chemical Co., St. Louis, MO). The stock solution of DFP was 0.01 mol/L in 0.1 mol/L NaOH. DFP stock solutions are stable at 4°C for at least 6 months. Alkaline phosphatase-labeled goat anti-rabbit IgG (GARIg-ALP) and sheep anti-mouse immunoglobulin G (Fc fragment-specific) were obtained from Jackson

Immunoresearch, West Grove, PA. Working solutions of GARIg-ALP were prepared by diluting the stock solution 3,000-fold in the assay buffer (described below). White, opaque 12-well polystyrene microtiter strips were obtained from Dynatech Labs., Alexandria, VA. The substrate buffer was a Tris buffer (0.1 mol/L, pH 9.1) containing 0.1 mol of NaCl and 1 mmol of $MgCl_2$ per liter. The substrate working solution (DFP, 1 mmol/L in substrate buffer) was prepared just before use by diluting the DFP stock solution 10-fold in the substrate buffer. The wash solution was prepared by dissolving 9 g of NaCl and 0.5 g of polyoxyethylenesorbitan monolaurate (Tween 20) in 1 L of a 10 mmol/L Tris buffer, pH 7.40. The developing solution contained 1 mol of Tris base, 0.4 mol of NaOH, 2 mmol of $TbCl_3$ and 3 mmol of EDTA per liter (no pH adjustment). The assay buffer A was a 50 mmol/L Tris buffer, pH 7.40, containing 60 g of BSA, 0.5 g of sodium azide, 100 mL of normal goat serum, 25 mL of normal mouse serum, 5 g of bovine IgG and 0.5 g of Tween 20 per liter. The assay buffer B was the same as assay buffer A except that mouse serum was omitted.

CLINICAL SAMPLES

Several clinical samples were used to examine the presence of hK6. These included serum and urine samples from male and female individuals (healthy blood donors), breast cyst fluids obtained by needle aspiration, breast tumor cytosolic extracts, prepared as described previously (*11*), amniotic fluids, milks from lactating women, seminal plasmas, nipple aspirate fluids (NAFs) and cerebrospinal fluids (CSFs). In addition, a panel of human tissue cytosolic extracts, prepared as previously described were tested (*Hassapoglidou, S. et al Oncogene 1993, 8:1501-1509.*). To establish optimal measuring conditions, all samples were tested at various dilutions. The procedures are in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983.

All tissues and fluid samples were stored at -80°C until use.

INSTRUMENTATION

A time-resolved fluorometer, the CyberFluor 615 Immunoanalyzer (MDS Nordion, Kanata, ON, Canada) was used to measure Tb^{3+} fluorescence in white microtiter wells. This procedure has been described in detail elsewhere (*Christopoulos, TK, et al Anal Chem 1992, 64:342-346; Ferguson RA et al, Clin Chem 1996 42: 675-684*).

PROCEDURES

Production and purification of recombinant hK6 protein. Human 293 cells transfected with a plasmid containing the 1.4-kb hK6 cDNA were subjected to selection by growth in G418 (400 mg/L) for three weeks, after which time stable transformants were isolated. One clone generated identifiable amounts of hK6 protein in the culture medium. This cell line was cultured and the tissue culture supernatant was collected and concentrated by using Centricon ultrafiltration devices (Millipore, Waltham, MA 02454). Purification of hK6 from the concentrated cell culture supernatants was achieved by reversed-phase high pressure liquid chromatography (C-8, Aquapore RP-300, 0.45 x 25 cm, Applied Biosystems, Foster City, CA) using a linear gradient of 0.1% trifluoroacetic acid/acetonitrile. Generally, the gradient increased at a rate of 1% acetonitrile per min. Factions containing hK6 were located by SDS-polyacrylamide gel electrophoresis,

collected, lyophylized and stored at -20°C (Little SP et al, J. Bil Chem 1997:272:251135-25142).

Development of polyclonal antibodies against hK6. Purified recombinant hK6 protein was used to immunize rabbits and mice using standard procedures (*Campbell Am, Production and purification of antibodies. In: Immunoassay. Diamandis EP Christopoulos TK (eds)*00. 95-115, Academic Press, San Diego, 1996). The rabbit and mice antisera were used for the development of the immunofluorometric assay without further purification.

Coating of microtiter plates with sheep anti-mouse immunoglobulin. White polystyrene microtiter wells were coated by incubating overnight 500 ng / 100 µL per well of the coating antibody diluted in a 50 mmol/L Tris buffer, pH 7.80. The wells were then washed six times with the wash solution and blocked for 1 hour with 200 µL/well of the blocking solution (10 g/L BSA in 50 mmol/L Tris, pH 7.80). After another six washes, the wells were ready to use.

hK6 calibration. hK6 calibrators of 0, 1, 5, 20, 50 and 200 µg/L were prepared by diluting recombinant purified hK6 protein in a 50 mmol/L Tris buffer, pH 7.80, containing 60 g of BSA and 0.5 g of sodium azide per liter.

hK6 assay. Calibrators or samples (100 µL) were pipetted into the microtiter wells and 50 µL of the polyclonal mouse anti-hK6 antiserum, diluted 5,000-fold in assay buffer B, were added. The wells were then incubated with shaking at room temperature for 2 hours and washed six times. To each well, was added 100 µL of rabbit anti-hK6 antibody, diluted 1,000-fold in assay buffer A, incubated for 30 min as described above, and then washed six times. To each well, was added 100 µL of a goat anti-rabbit immunoglobulin, conjugated to alkaline phosphatase, diluted 3,000-fold in assay buffer A and incubated for 30 min, as described above. The wells were then washed six times; 100 µL of 1 mmol/L DFP working substrate solution was added, and the wells were incubated for 10 min, as described above. 100 µL of developing solution was added to each well, the wells were mixed by mechanical shaking for 1 min and the fluorescence was measured with the time-resolved fluorometer. The calibration and data reduction were performed automatically by the CyberFluor 615 Immunoanalyzer.

***High performance liquid chromatography (HPLC)*:** Various biological fluids have been fractionated on a gel filtration column, using the procedures described elsewhere (*Yu H, Diamandis EP, Clin Chem 1993: 39:2108-2114; Diamandis, EP at al Cliln Chem 1997:43:1365-1371)*). HPLC fractions were collected and analyzed for hK6 with the developed immunofluorometric assay.

Results

ASSAY OPTIMIZATION

Two polyclonal antibodies against recombinant hK6 protein were used, one developed in mice and one developed in rabbits. The chosen assay configuration (indirect coating of the wells with a sheep anti-mouse antibody and detection of the immunocomplex with a goat anti-rabbit immunoglobulin, conjugated to alkaline phosphatase) demonstrated good sensitivity (see below) without the need for any purification or conjugation of the primary antibodies. The amounts of antibodies used, the diluents and incubation times of the various assay steps were optimized. Optimal conditions were selected based on the lowest achievable

detection limit and best assay linearity and dynamic range. The final conditions are described above.

CALIBRATION CURVE, DETECTION LIMIT, PRECISION

A typical calibration curve of the proposed hK6 assay is shown in Figure 1. The detection limit, defined as the concentration of hK6 corresponding to the fluorescence of the zero calibrator plus two standard deviations, is ≤ 0.5 μg/L. Within-run and between-run precision was assessed at various hK6 concentrations between 2-50 μg/L and with various clinical samples. In all cases, the coefficients of variation (CVs) were between 2 and 9%, consistent with the precision of typical microtiter plate-based immunoassays.

SPECIFICITY

hK6 protein was detected in various biological fluids. In order to ensure that the immunofluorometric assay measures hK6 with high sensitivity and specificity, separated in a gel filtration column three biological fluids with relatively high hK6 concentration, (namely one human milk from a lactating woman, one cerebrospinal fluid and one serum sample from an ovarian cancer patient who was found to have high levels of this biomarker in serum) were separated in and measured on a gel filtration column. The results are shown in Figure 2. In all three biological fluids tested, a single immunoreactive species of a molecular mass of ~ 30 kDa was detected, which is consistent with the molecular mass of hK6 protein. Higher molecular weight complexes were not detected suggesting that hK6 is present in these biological fluids in its free form. Other serum proteinases (e.g. PSA) are present in serum and other fluids mostly bound to proteinase inhibitors (Stenman U-H, et al, Cancer Res. 1991: 51:222-226); Christensson A et al Cur J Biochem 1990; 194; 755-763).

hK6 IN BIOLOGICAL FLUIDS AND TISSUE EXTRACTS

To obtain preliminary information on the presence of hK6 in biological fluids, various clinical samples were analyzed, as shown in Table 1. The highest concentration of hK6 was found in milk of lactating women, followed by cerebrospinal fluid, nipple aspirate fluid and breast cyst fluid. hK6 was also detected in male and female serum samples, in the majority of seminal plasmas and in a relatively small percentage of amniotic fluids and breast tumor cytosolic extracts. hK6 protein was not detected in urine.

A number of human tissue cytosolic extracts were also tested. The highest concentration of hK6 was detected in the salivary glands, followed by lung, colon, fallopian tube, placenta, breast, pituitary and kidney. The following tissues tested negative: skin, spleen, bone, thyroid, heart, urerter, liver, muscle, endometrium, testis, pancreas, seminal vesicle, ovary, adrenals and prostate (Figure 3).

Discussion

The present inventors have developed polyclonal antibodies and an immunofluorometric procedure suitable for quantifying hK6 protein in biological fluids and tissue extracts. Since a rich natural source of hK6 protein is not known, recombinant hK6 protein was used for the development of polyclonal rabbit and mice antibodies. This recombinant protein ensures high purity without any contaminating proteins. The chosen assay configuration does not need any further purification or conjugation of the primary antibodies used, and it is thus a convenient method for developing sensitive immunofluorometric procedures. The same principle has been adopted previously for measuring the p53 tumor suppressor in biological fluids

(Hassapoglidou S et al, Oncogene 1993: 8: 1501-1509).

The developed immunoassay for hK6 protein demonstrates good sensitivity, dynamic range and linearity (Figure 1). It has been further verified that this assay detects a single immunoreactive band in the biological fluids examined. In serum, this proteinase is present in its free form, similarly to observations with hK2 measurements (Black, MH et al Clin Chem 1999; 45:790-799). However, this is in contrast to the situation with PSA, which is known to be present in serum mainly bound to α_1-antichymotrypsin (Stenman U-H, et al, Cancer Res. 1991: 51:222-226); Christensson A et al Cur J Biochem 1990; 194; 755-763).

The survey of a relatively large number of biological fluids has indicated that hK6 protein is present at relatively high concentrations in milk of lactating women and other breast secretions, including nipple aspirate fluid and breast cyst fluid (Table 1). Previously, the presence of other kallikreins, including PSA and hK2, has been demonstrated in these biological fluids (*Yu, H Diamandis; Clin Chem 1995: 41:54-58; Sauter ER et al Cancer Epidemiol Biomarkers Prevent 1996 967-970; Diamandis Ep et al Breast Cancer Res Treat 199638:259-264; Balck MH et al Br J Cancer 2000; 82:361-367; Blcak MH et al Clin Chem 1999;45: 790-799; yu H. and Diamandis EP Clin Chem 1995:41:204-210; Black MH Diamandis EP, Breast Cancer Res Treat 2000 59:1-14).* Large amounts of hK6 protein were detected in cerebrospinal fluid, which are consistent with the observation that hK6 is expressed at high levels in brain tissue (Little, supra). hK6 was also found in male and female sera and seminal plasmas and in a small percentage of amniotic fluids and breast tumor cytosols. Previously, PSA and hK2 was demonstrated in these biological fluids as well (*Yu, H Diamandis; Clin Chem 1995: 41:54-58; Sauter ER et al Cancer Epidemiol Biomarkers Prevent 1996 967-970; Diamandis Ep et al Breast Cancer Res Treat 199638:259-264; Balck MH et al Br J Cancer 2000; 82:361-367; Blcak MH et al Clin Chem 1999;45: 790-799; yu H. and Diamandis EP Clin Chem 1995:41:204-210; Black MH Diamandis EP, Breast Cancer Res Treat 2000 59:1-14*). It is interesting to note that although seminal plasma contains extremely high levels of PSA and hK2 (Diamandis EP Trends Endocrinol Metab 1999: 25:14-26' RittenhouseHe et al Crit Rev Clin Lab Sci 1998: 35:275-368), the assay described herein detected very small amounts of hK6 in this biological fluid (Table 1). This further demonstrates that the homologous proteins PSA and hK2 do not have any major cross-reactivity with the developed hK6 assay.

The assay developed here represents the first method for detecting hK6 protein in biological fluids. The results further demonstrate that hK6 is a secreted protein, as predicted by its deduced amino acid sequence (Yousek GM et al Genomics 1999;62:251-259).

Example 2

Materials and Methods

Immunofluorometric assay for hK6

The details of this immunofluorometric assay have been described (See Example 1 and ref. 12). The assay utilizes two hK6-specific polyclonal antibodies, one raised in mouse and the other raised in rabbit. This is a non-competitive immunofluorometric procedure which incorporates the principles of time-resolved fluorometry for detection. The assay measures hK6 in the range of 0.5-200 μg/L with precision < 10%.

Serum samples were analyzed without sample pretreatment.

Clinical samples

For this investigation, leftover serum samples obtained from patients with various malignancies were used (Table 2). Patients were included with relatively high tumor burden (as indicated by tumor marker levels of at least 10-fold higher than the upper limit of normal) in order to increase the chance of detecting possible hK6 elevations in serum. All serum samples were stored at -20°C until analysis for a maximum time of one year. The procedures are in accordance with the Ethical Standards of the Helsinki Declaration of 1975, as revised in 1983.

Analysis of tumor markers

The tumor markers CA125, PSA, CEA and AFP were analyzed on the Elecsys immunoassay analyzer (Roche Diagnostics, Indianapolis, IN). CA15.3, CA19.9 and hCG were analyzed on the Immuno 1 immunoassay analyzer (Bayer Diagnostics, Tarrytown, NY) and calcitonin was measured with a radioimmunoassay kit from Diasorin, Italy. The upper limit of normal values for the tumor markers were 35 KU/L (CA125), 4 µg/L (PSA), 10 µg/L (AFP), 5 µg/L (CEA), 35 KU/L (CA15.3), 37 KU/L (CA19.9), 10 IU/L (hCG) and 100 ng/L (calcitonin).

Results

A total of 378 serum samples were analyzed with the previously described immunofluorometric assay for hK6 (12). These samples were from either normal individuals (male and female) or from patients with various malignancies. The obtained data are shown in Table 2. While in none of the normal controls and in only two samples from patients with non-ovarian malignancies the hK6 concentration was above 15 µg/L (at arbitrary cutoff), the majority of patients with ovarian carcinoma (~ 66%) had highly elevated hK6 concentrations in their serum (>15 µg/L). The distribution of hK6 values in serum of ovarian cancer patients is shown in Figure 4. As shown in Figure 5, the correlation between hK6 concentrations and CA125 levels is poor and not statistically significant.

In Figure 6, data is presented on temporal changes of serial serum hK6 and CA125 concentration in four patients with ovarian cancer. The hK6 concentration changes during the monitoring period, similarly to CA125, suggesting that this new biomarker may have value for patient management.

Discussion

The data of Table 2 summarize the findings and demonstrate that among all cancer types tested (normal males and females versus breast, thyroid, testicular, gastrointestinal, prostate, lung and ovarian cancer), only ovarian cancer patients show significantly elevated levels of this biomarker in the circulation. Approximately 66% of patients had levels higher than 15 µg/L, a cutoff that affords 98-100% specificity for all other cancers tested. Although these data are highly promising, regarding value of hK6 as a circulating biomarker for ovarian carcinoma, it should be taken into consideration that all patients with ovarian cancer had relatively high levels of CA125 (≥ 372 KU/L, which is approximately 10 times higher than the upper reference range). The data of Figure 6 indicate that serum levels of hK6 change with time during ovarian cancer monitoring, suggesting that this biomarker may be useful for monitoring patients after primary

treatment.

As is evident from Figure 5, there is no significant correlation between hK6 concentration and CA125, suggesting that these two biomarkers may be complementary for the diagnosis and management of ovarian carcinoma.

In conclusion, the first evidence that serum hK6 concentration is significantly increased in about 66% of ovarian cancer patient is provided. The test seems to be specific for ovarian cancer since no such increases were seen in various other malignancies. Therefore, hK6 represents a novel serum biomarker for ovarian cancer which may be useful for disease diagnosis and monitoring.

Example 3

Materials and Methods

Patient population

Included in this study were 97 apparently healthy women (ages 26 to 72 years; mean = 52, median = 49 years), 141 women with benign disease (ages 21 to 76 years; mean = 46, median = 45 years) and 146 patients with histologically proven primary ovarian carcinoma (ages 28 to 78 years; mean = 56, median = 57 years). Of the benign lesions, 50 were classified as endometriosis, 22 as mucinosum, 10 as ovarian teratomas, 26 as dermoidea, 15 as corpus luteum and 18 as serosum. Tumors were staged according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. Histologic classification was based on the World Health Organization and FIGO recommendations. The characteristics of the ovarian cancer patients in terms of stage, grade, histotype, residual tumor post-surgery, debulking success and response to chemotherapy are shown in Table 7. Serum samples from all patients were collected pre-surgically, before initiation of therapy, and stored at -80°C until analysis. For 105 ovarian cancer patients, serum was also available post-surgery. This sample was obtained approximately 2-3 weeks post-surgery.

Sera were obtained from four centres as follows: The Gynecologic Oncology Unit, University of Turin, Italy (97 normals, 14 benign, 21 cancers); Holland (40 cancers); Belgium (13 benign, 85 cancers); Department of Clinical Chemistry, Helsinki University Central Hospital, Finland (114 benign).

Patients were monitored for survival and disease progression for a median duration of 25 months (range 1-106 months). Follow-up information was available for 131 of the ovarian cancer patients. Sixty-four (49%) of these relapsed and 28 (21%) died during the course of the follow-up period.

Analysis of hK6 and CA125

CA125 was measured with a commercially available automated immunoassay method (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA). The upper limit of normal for this method is 23 KU/L. The concentration of hK6 was measured with a procedure described herein (12) with some modifications. This assay employs a monoclonal anti-hK6 mouse antibody, coated directly on microtiter wells (capture antibody), a polyclonal rabbit detection antibody and an alkaline phosphatase-conjugated goat anti-rabbit antibody. Signal was quantified by time-resolved fluorometry. The assay has a detection limit of 0.1 µg/L and a dynamic range up to 50 µg/L. Precision was <10% within the measurement range. The serum samples were analyzed in duplicate with inclusion of three quality control samples in every run.

Statistical analysis

To analyze data, patients were divided into different groups according to clinical and pathological parameters. The analyses of differences between hK6 serum concentration before and after surgery were performed with the non-parametric McNemar test. The binomial distribution was used to compute the significance level of the McNemar test.

Receiver operating characteristic (ROC) curves were constructed for hK6 and CA125 serum concentration by plotting sensitivity versus (1-specificity) and the areas under the ROC curves (AUC) were calculated. The non-cancer group included the normal individuals and the patients with benign disease. Correlations between different variables were assessed by the Spearman correlation coefficient. The non-parametric Mann-Whitney U test was used to determine differences between two groups and the non-parametric Kruskal-Wallis test was used for the analysis of differences among more than two groups. These tests treated hK6 concentration in serum as a continuous variable. hK6 serum concentration was also classified as either hK6-positive (> 4.4 μg/L) or hK6-negative (≤ 4.4 μg/L). The relationship of this dichotomous variable with other clinicopathological correlates was established with the Chi Square (χ^2) test or the Fisher's Exact test, as appropriate.

Kaplan-Meier progression-free survival and overall survival curves were constructed to demonstrate the survival differences between the hK6-positive and hK6-negative patients. The log rank test was used to examine the significance of the differences among the survival curves. The impact of serum hK6 concentration on patient overall survival (OS) and on progression of the disease (progression-free survival; PFS) was assessed with the hazards ratio, calculated by both univariate and multivariate Cox proportional hazards regression models. In the multivariate analysis, the clinical and pathological variables that may affect survival, including stage of disease, tumor grade, residual tumor and histologic type were adjusted.

Results

Serum hK6 concentration in cancer and non-cancer patients: The mean, median, range and selected percentiles of serum hK6 concentration among non-cancer (normal; n = 97), benign disease (n = 141), pre-surgical (n = 146) and post-surgical (n = 105) ovarian cancer patients is shown in Table 3. The mean and median values between non-cancer (normal) and benign disease patients were not statistically significant. The mean and median hK6 values in pre-surgical ovarian cancer patients were significantly higher than the non-cancer and benign groups ($p < 0.001$). The distribution of hK6 concentration in the three groups of patients (normal, benign, pre-surgical ovarian cancer) is further presented in Figure 7 along with the corresponding CA125 values. Clearly, pre-surgical serum hK6 concentration is not different between normal and benign disease patients but is significantly elevated in a proportion of ovarian cancer patients (Figure 7A). Conversely, CA125 values are progressively increased from normal, to benign, to cancer patients (Figure 7B).

For dichotomous classification of this patient population as hK6-positive and hK6-negative, the hK6 cutoffs of 4.2 μg/L (90% diagnostic specificity) and 4.4 μg/L (95% diagnostic specificity) were selected.

Changes of serum hK6 concentration post-surgery: For 105 patients with ovarian cancer, pre-surgical and

post-surgical serum samples were selected. As shown in Figure 8, 71 patients (68%) demonstrated a drop in hK6 concentration post-surgery, 21 (20%) had unchanged values and 13 (12%) had higher hK6 serum levels after the operation. By the McNemar test, the concentration drop post-surgery was statistically highly significant ($p < 0.001$).

Correlation between serum hK6 and CA125 concentration: The logarithmic plot of Figure 9 shows that there is a weak correlation between serum hK6 and CA125 concentration (Spearman correlation $r_s = 0.44$). While the correlation is significant, there are still many samples with quite variable values. For example, at CA125 levels around 500 KU/L, hK6 concentration ranges from 2-40 µg/L while samples with hK6 levels around 6 µg/L may have CA125 values ranging from 5 to > 5,000 KU/L.

Diagnostic sensitivity and specificity of serum hK6 concentration: For this calculation, the various subgroups of patients were considered, as shown in Table 4. In the non-cancer group, all patients who are either normal or have benign disease were included. When the whole patient group was analyzed, diagnostic sensitivity is around 54% at 90% specificity and 50% at 95% specificity. The receiver operating characteristic (ROC) curve of Figure 10 indicates a slight diagnostic advantage of CA125, in comparison to hK6. However, the two markers can work in combination, since hK6 concentration could be elevated in a subset of patients with relatively low CA125. In the subgroup of patients with CA125 > 60 KU/L, the diagnostic sensitivity of hK6 is 71 and 65% at specificities of 90 and 95%, respectively. In the subgroup of patients with low CA125 (< 23 KU/L), about 13-17% of patients will still have elevated hK6, at hK6 cut-offs of 4.4 (95% specificity) or 4.3 µg/L (90% specificity), respectively. In the subgroup of patients with slightly elevated CA125 (23-60 KU/L), the diagnostic sensitivity of hK6 is 15-26% at specificities of 95-90%, respectively (Table 4).

In Table 5, the additional contribution of hK6 in identifying ovarian cancer patients was calculated by using either CA125 alone or CA125 plus hK6. Among all patients with known stage (N = 124), hK6 analysis increases the sensitivity of CA125 by 12% or 13%, at 90% or 95% specificity cut-offs for both markers. The contribution is still significant at ovarian cancer stages I/II (43 patients). The addition of hK6 increases the sensitivity of CA125 alone from 30% to 42%, or from 26% to 37%, at 90% or 95% specificity cut-offs for both markers, respectively.

Table 6 summarizes the relative risk (RR) of having ovarian cancer, based on serum hK6 concentration. The relative risk increases exponentially with increasing hK6 concentration, reaching a value of 20 when hK6 is ≥ 4.3 µg/L. The RR is still substantial (RR = 5.3) in multivariate analysis, after adjusting for CA125 levels.

Prognostic value of serum hK6: Higher ovarian cancer stage and grade are strongly associated with higher serum hK6 concentration (Figure 11 and Table 7). Furthermore, serous adenocarcinomas are more frequently associated with high serum hK6 concentration (positivity 68%) followed by endometrioid tumors (positivity 33%); mucinous tumors are rarely associated with high serum hK6 (9%). Furthermore, high serum hK6 concentration is associated with presence of residual tumor, suboptimal debulking and poor response to chemotherapy. All these associations were highly significant ($p < 0.001$).

In univariate Cox analysis, serum hK6 concentration is associated with shorter progression-free and

overall survival (Table 8). These associations remained statistically significant in the multivariate analysis. The prognostic value of CA125 was no longer statistically significant in the multivariate analysis. Besides pre-surgical serum hK6, stage of disease was the only other parameter that was associated with both progression-free and overall survival in multivariate analysis (Table 6).

Similar data were obtained with Kaplan-Meier survival analysis (Figure 12). Patients with high pre-surgical serum hK6 have much shorter progression-free and overall survival than patients with low pre-operative hK6 levels. While virtually all patients with high serum hK6 relapsed by 6 years, more than 50% of patients with low pre-operative serum hK6 were still in remission.

Discussion

The discovery of new ovarian cancer biomarkers for early diagnosis, prognosis, monitoring and prediction of therapeutic response will likely contribute to improved clinical outcomes. The only well accepted ovarian cancer biomarker, CA125, was discovered 20 years ago. A number of other potential ovarian cancer biomarkers have been identified but their clinical value is not established (27). A novel ovarian cancer biomarker, human kallikrein 6 (hK6), a member of the expanded human kallikrein gene family, is described herein.

The traditional ovarian cancer biomarker, CA125, falls short of being able to diagnose early ovarian cancer. In addition to its low sensitivity for early disease, CA125 also suffers from low specificity i.e. elevated levels are seen in many benign abdominal diseases. Currently, it is widely accepted that no single cancer biomarker will provide all the necessary information for optimal cancer diagnosis and management. The current trend is to focus on the identification of multiple biomarkers which can be used in combination. Such approaches have already shown to have clinical potential in ovarian cancer (28,29).

Serum hK6 represents a novel biomarker for ovarian carcinoma. This biomarker is more specific for ovarian cancer than CA125 since, in contrast to CA125, elevations were not seen in benign diseases (Figure 7). The diagnostic sensitivity of hK6 is slightly less than the diagnostic sensitivity of CA125 at the same specificity cut-offs (Table 5 and Figure 10). However, hK6 can increase the diagnostic sensitivity of CA125 at all stages of the disease, including stage I/II disease (Table 5). Despite the weak correlation between hK6 and CA125 (Figure 9), there are still patients with normal CA125 who have elevated hK6 levels (Table 4). Thus, CA125 and hK6 could be used in combination to increase the diagnostic sensitivity of each of the biomarkers alone.

Similarly to the situation with CA125, hK6 concentration is more frequently elevated in serous ovarian carcinoma than in endometrioid and mucinous carcinomas (Table 7). Serum hK6 concentration is also more frequently elevated in late stage and higher grade disease. Serum hK6 concentration is a powerful predictor of patient outcomes. Patients with pre-operative hK6 concentration above 4.4 μg/L have significantly worse prognosis than patients with low pre-operative hK6 (Table 8 and Figure 12). Serum hK6 concentration is a more powerful prognostic indicator that serum CA125. The prognostic value of CA125 disappears in multivariate analysis while serum hK6 is an independent prognostic indicator, as shown in the multivariate analysis of Table 8. Serum hK6 likely originates from tumor cells, since post-operatively, the

levels are significantly decreased (Figure 8). In the study in Example 4 examining the prognostic value of hK6 analysis in ovarian tumor extracts, the overexpression of hK6 in tumor cells was verified by immunohistochemistry and further provided evidence that intratumor hK6 concentration is also a strong predictor of prognosis. Interestingly, many other members of the human kallikrein gene family, including the enzymes hK4, hK5, hK7, hK8, hK9 and hK10 have already shown to have prognostic significance in ovarian cancer (34-41). Serine proteases not belonging to the kallikrein family have also been shown to have prognostic significance in ovarian cancer, including trypsin, hepsin and testisin (42-44). Yet, it has been known for years that many other proteolytic enzymes have prognostic value in many cancers (for reviews, see 45 and 46). The biological mechanisms of proteolytic enzyme involvement in cancer prognosis includes their ability to degrade extracellular matrix, thus facilitating invasion and metastasis (47-49). It seems likely that multiple members of the human kallikrein gene family are disregulated in ovarian cancer. It is thus possible that other members of this protease family may emerge as potential ovarian cancer biomarkers. If these proteases are involved in cancer progression, they may be suitable candidates as therapeutic targets.

Table 7 shows preliminarily that pre-surgical serum hK6 concentration may be a predictor of response to chemotherapy in ovarian cancer patients. Among the non-responders, 81% had elevated pre-surgical hK6 concentration while 19% of these patients had low hK6 concentration. Among the patients who had either complete or partial response to chemotherapy, 57% had low pre-operative hK6 concentration ($p < 0.001$).

In conclusion, serum hK6 concentration represents a novel biomarker for ovarian carcinoma, which has potential utility as a diagnostic, prognostic and predictive tool. The combination of hK6 and CA125 improves the diagnostic sensitivity of ovarian cancer at all stages, including early stage disease.

Example 4

KLK6 Ovarian Tissue

PATIENTS AND METHODS

Ovarian Cancer Patients. One hundred eighty patients with primary ovarian cancer were included in this study. These patients underwent surgery for ovarian cancer at the Department of Gynecology, University of Turin, Italy. Patient age ranged from 25 to 82 years with a median of 59 years. Clinical and pathological information documented at the time of surgery included clinical stage of the cancer, grade and histology of the tumor, and amount of remaining tumor. Menopausal status was documented and response to chemotherapy monitored. Tumors were staged according to the International Federation of Gynaecology and Obstetrics (FIGO) criteria. Histologic classification was based on the World Health Organization and FIGO recommendations. Of the tumors included in this study, 80 were classified as serous papillary, 32 as undifferentiated, 27 as endometrioid, 13 as mucinous, 14 as clear cell, 10 as mullerian and 4 as other. The size of the residual tumors ranged from 0 to 9 cm, with a median of 1.1 cm.

Patients were monitored for survival and disease progression (no apparent progression or progression) for a median duration of 62 months (range 1-99 months). Follow-up information was available

for 165 of the patients. 97 (54%) of these relapsed and 61 (34%) died during the course of the follow-up period.

Investigations were carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983, and were approved by the Institute of Obstetrics and Gynecology, Turin, Italy.

Preparation of Tumor Cell Extracts. Tumor tissue was frozen in liquid nitrogen immediately after surgery and stored at -80^0C until extraction. 20 to 100 mg of frozen tissue was pulverized on dry ice to a fine powder and added to 10 volumes of extraction buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 10g/L of NP-40 surfactant, 1 mM phenylmethyl sulphonyl fluoride, 1g/L of aprotinin, 1g/L of leupeptin). The resulting suspension was incubated on ice for 30 minutes during which time it was vortexed every ten minutes. The mixture was then centrifuged at 14,000 rpm at 4^0C for 30 minutes and the supernatant (cell extract) was collected and stored at -80^0C until analysis. Protein concentration of the extract was determined with the bicinchoninic acid method, with albumin as standard (Pierce Chemical Co., Rockford, IL).

Measurement of hK6 in Ovarian Cell Extracts. The concentration of hK6 in tumor cell extract was quantified with a highly sensitive and specific non-competitive immunoassay for hK6 that has been previously described and evaluated in detail (12). The assay incorporated two hK6-specific polyclonal antibodies, one raised in mouse and the other in rabbit, in a sequential two site immunometric format with time resolved fluorescence detection. Analysis of standards, tumor cell extracts and control pools was carried out in duplicate in 96-well polystyrene microtiter plates with 200 µL of specimen added to the immunoassay. The standard curve using recombinant hK6 protein ranged from 0.5 µg/L to 200 µg/L. Assay precision was better than 10%. Signal detection and data reduction were performed automatically by the CyberFluor 615 Immunoanalyzer.

Localization of hK6 in Ovarian Tumor Specimens by Immunohistochemistry. A rabbit polyclonal antibody was raised against hK6 full-size recombinant protein, produced in yeast cells. Immunohistochemical staining for hK6 was performed according to a standard immunoperoxidase method. Briefly, paraffin-embedded tissue sections (4 µm) were fixed and dewaxed. Endogenous peroxidase activity was blocked with 3% aqueous hydrogen peroxide for 15 minutes. Sections were then treated with 0.4% pepsin at pH 2.0 for 5 minutes at 42^0C and blocked with 20% protein blocker (Signet Labs) for 10 minutes. The primary antibody was then added at 1:400 dilution for 1 hour at room temperature. After washing, biotinylated anti-rabbit antibody (Signet) was added, diluted 4-fold in antibody dilution buffer (DAKO). Following incubation and washing, streptavidin tagged horseradish peroxidase was added for 30 minutes at room temperature. After washing, detection was achieved with amino ethyl carbazole (AEC) for 5-10 minutes. The slides were counterstained with hematoxylin and then mounted with cover slips.

Statistical Analysis. Statistical analysis was performed with SPSS software (SPSS Inc. Richmond, CA). To analyze data, patients were divided into different groups according to clinical and pathological parameters. Because the distribution of hK6 mass per mg total protein (i.e. specific activity) in the ovarian tumor extracts was not Gaussian, the non-parametric Mann-Whitney U test was used to determine differences between two groups and the non-parametric Kruskal-Wallis test was used for the analysis of differences among more than

two groups. These tests treated hK6 specific activity in the tumor extract (ng hK6/mg total protein) as a continuous variable. hK6 tumor extract specific activity was also classified as either hK6-positive (> 35 ng/mg total protein; see Figure 7B for explanation) or hK6-negative (≤ 35 ng/mg total protein). The relationship of this dichotomous variable to other clinicopathological correlates was established with the Chi Square (χ^2) test or the Fisher's Exact Test, as appropriate. The impact of tumor extract hK6 specific activity on patient survival and on progression of the disease (progression-free survival) was assessed with the hazards ratio calculated by both univariate and multivariate Cox proportional hazards regression models (30). In the multivariate analysis, the clinical and pathological variables that may affect survival, including stage of disease, tumor grade, residual tumor, histologic type and age were adjusted. Kaplan-Meier progression-free survival and overall survival curves (31) were constructed to demonstrate the survival differences between the hK6-positive and hK6-negative patients. The log rank test (32) was used to examine the significance of the differences among the survival curves. Following analysis of the entire patient data set as a whole, the process was repeated on subgroups stratified separately by disease stage, by tumor grade and by amount of tumor remaining following surgery (debulking success). The impact of tumor hK6 level (positive or negative) on survival and on disease progression was determined by univariate and multivariate models for each of the subgroups.

RESULTS

Distribution of hK6 Specific Activity in Ovarian Tumor Extracts. The distribution of hK6 specific activity in ovarian tumor extracts from the 180 patients (Figure 13A) ranged from 0.04 ng/mg total protein to 497 ng/mg of total protein with a mean of 33 ng/mg total protein and a median of 13.2 ng/mg total protein. A value of 35 ng/mg total protein was identified by Chi square analysis ($\chi^2 = 7.3$; $P = 0.007$) as the optimal cutpoint to distinguish positive from negative tumors in terms of predicting overall survival (Figure 13B). Thirty percent of the tumors were hK6 positive by this criterion. hK6 specific activity in tumor extracts was treated both as a continuous variable and as a dichotomous variable (≤ 35 ng/mg total protein, > 35 ng/mg total protein) in the analyses that follow.

hK6 specific activity (ng hK6/mg total protein) was significantly elevated ($P < 0.001$ by the Kruskal Wallis test) in extracts of ovarian tumor (mean 32.7, standard error 3.8, range 0.04 to 497) compared to extracts prepared from normal ovarian tissues (mean 3.5, standard error 2.5, range 0.05 to 20.8) or from ovarian tissue with benign disease (mean 3.2, standard error 2.6, range 0.03 to 21.5) (Figure 14). Further analysis showed there was no significant difference in hK6 specific activity among the ovarian tumors when they were stratified by histotype (i.e. serous vs undifferentiated vs endometrioid, etc) (data not shown).

Relationships between hK6 Status and Other Clinicopathological Variables. The distributions of various clinicopathological variables between hK6-positive and hK6-negative patients are summarized in Table 9. The relationships between hK6 status and these variables were examined with either the χ^2 Test or Fisher's Exact Test, as appropriate. No relationship was observed between hK6 status and tumor grade, menopausal status and response to chemotherapy. However, hK6-positive patients were more likely to have advanced disease (stage II-IV), serous tumor histology and greater residual tumor (>1 cm) (all $P<0.05$). hK6 tumor

extract specific activity when treated as a continuous variable also associated proportionally with stage of the disease. Figure 15 shows the distribution of hK6 specific activity stratified by disease stage. hK6 specific activity was significantly higher in extracts from stage III/IV ovarian cancer than in those from stage I/II (P = 0.002 by the Mann Whitney U Test).

Univariate and Multivariate Survival Analysis. The impact of hK6 specific activity, other clinicopathological variables and age on disease progression and on overall survival is presented in Table 10. In univariate analysis, hK6-positive patients had a significantly increased risk of disease progression (hazard ratio =1.71) and death (hazard ratio =1.88) (P<0.05). When hK6 specific activity was treated as a continuous variable, hazard ratios were closely similar to those of hK6 negative tumors (arbitrarily set at 1.00), although the slight increase in risk of disease progression (hazard ratio = 1.005) was highly significant at P = 0.001. Kaplan-Meier survival curves demonstrated survival differences between hK6-positive and hK6-negative patients. As Figure 16 shows, the probability of progression-free and overall survival, respectively, are lower in hK6-positive patients than in hK6-negative patients.

The adverse effects of hK6 positivity on progression free survival and on overall survival were lost in multivariate analysis. As shown in Table 10, when survival outcomes were adjusted for other clinicopathological variables, hK6-positive and hK6-negative patients had statistically similar rates of disease progression and overall survival. Tumor grade also lost its univariate prognostic significance in multivariate analysis. Only stage of disease and residual tumor remaining after surgery maintained their independent effects on survival outcome in the multivariate analysis.

Univariate and Multivariate Survival Analysis in Subgroups of Patients. The patients were divided into different subgroups based on disease stage, tumor grade, and debulking success (residual tumor). In each subgroup, the impact of hK6 positivity and negativity on disease progression and on overall survival was determined by univariate and by multivariate Cox proportional hazard regression models. The results are shown in Table 11. hK6 specific activity (positive, negative) significantly impacted survival in the subgroup of patients with tumor grade I or II. Univariate analysis revealed that hK6-positive patients were about 9-times more likely to suffer disease progression and 5-times more likely to die than hK6-negative patients. These survival differences remained significant even after the data were subjected to multivariate analysis. The relative risk of both outcomes arising from hK6 positivity was now about 4-fold (P < 0.03). hK6 status had no such effect among patients with Grade III tumor, nor could any discernible effect be demonstrated among patients with early stage disease and among those with greater than 1 cm of tumor remaining following surgery. Univariate analysis revealed a 2-fold increase in risk of disease progression and of death in the subgroup of patients with advanced disease (stage III and IV) who were hK6 positive, but the effect was lost in the multivariate analysis. The opposite occurred in the subset of patients characterized by optimal debulking of the tumor at the time of surgery (remaining tumor less than 1 cm in diameter). hK6 positivity had no demonstrable adverse effect on disease progression or on survival by univariate analysis, but did become statistically significant, giving a 3.5 and 5.5-fold increase in adverse risk, respectively, when the data were subjected to multivariate analysis. The emergence of effects in the multivariate model when none are

generated by the univariate model happens when the adjusted variables have no impact at all on the outcome. In the case here, this means that stage of disease, tumor grade, tumor histology and patient age had no prognostic potential on disease progression and overall survival in this particular subset of patients. Kaplan-Meier survival curves of the subset of patients with grade I or II ovarian tumor are shown in Figure 17. As expected from the univariate analysis mentioned earlier, there was a significant difference in disease progression and survival between hK6 positive and hK6 negative patients.

Immunohistochemical Staining of hK6 in Ovarian Tumors. Immunohistochemical staining of hK6 in paraffin embedded tumor sections was roughly proportional to hK6 specific activity in tumor extracts (data not shown). The immunohistochemical localization of hK6 protein in four ovarian tissues that contained benign, borderline or malignant tumor is depicted in Figure 18. hK6 staining was restricted to epithelial cells, being absent in mesenchymal elements including fibrous supporting stroma. hK6 stained within the cytoplasm of epithelial cells, but staining intensity was variable among and within tumor preparations.

DISCUSSION

Increased hK6 synthesis was found to be predictive of more aggressive tumor behavior over time. Considered apart from other clinicopathological variables and age, hK6 positivity across the entire patient population under study was associated with about a 2-fold increase in the risk of both disease progression and of death. This effect was lost when outcomes were adjusted for the other clinicopathological variables and age in multivariate analysis of the entire patient population, but not when the multivariate analysis was restricted to those patients with lower grade tumor and with less residual tumor remaining after surgery (<1 cm in diameter). Among the former subgroup of patients, hK6 positivity predicted about a 4-fold increase in the risk of disease progression and of death ($P < 0.03$) while corresponding hazard ratios in the latter subgroup were 3.75 and 5.5, respectively ($P < 0.02$). The data show that hK6 positivity has independent predictive potential in these two subgroups and gives insight into tumor behavior over time that cannot be gleaned from the clinical parameters and pathological correlates conventionally measured. Hence hK6 testing could contribute to more individualized effective treatment of such patients.

hK6 was found to be frequently overexpressed in ovarian tumors compared to nonmalignant ovarian tissue. This overexpression tended to be higher in tumors from late stage disease than from early stage disease. The histochemical studies suggest that hK6 is synthesized by the epithelial cells of the ovary and is distributed diffusely within the cytoplasmic compartment.

Epithelial ovarian cancer has one of the worst prognoses among gynecologic malignancies, largely because over three-quarters of the diagnoses are made at a time when the disease has already established regional or distant metastases (33). Compounding the problem, tumor progression and aggressiveness correlate variably with conventional clinical and pathological markers. Thus there is an important need for additional diagnostic and prognostic markers for this disease and a number of potential markers have been identified.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples.

To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Below full citations are set out for the references referred to in the specification.

Table 1 – Analysis of hK6 protein in various fluids.

Table 1: Analysis of hK6 protein in various fluids

Sample	hK6, µg/L			N[2]	Positivity rate
	Range	Mean (SD)	Median		(%)
Milk[1]	398 – 7,638	2,588 (1,607)	2,531	20	100
Cerebrospinal fluid (CSF)	41 – 2,053	605 (485)	525	21	100
NAF (normal)[3]	-	914	-	1 (pool)	100
NAF (cancer)[4]	-	737	-	1 (pool)	100
Breast cyst fluid	34 – 97	74 (25)	84	5 (pools)	100
Male serum	2.0 – 12.6	6.9 (2.6)	6.7	18	100
Female serum	0 – 8.1	4.1 (2.0)	4.4	18	100
Seminal plasma	0 – 17.7	6.8 (5.5)	5.0	16	81
Amniotic fluid	0 – 9.5	1.1 (2.2)	0	21	33
Breast tumor cytosols	0 – 33	2.1 (7.0)	0	36	17
Urine	0	0	0	10	0

1. From lactating women
2. Number of samples tested
3. Nipple aspirate fluid
4. NAF obtained from patients with breast cancer

Table 2 Concentration of human kallikrein 6 (hk6) in serum of normal individuals and patients with various malignancies.

Table 2 Concentration of human kallikrein 6 (hk6) in serum of normal individuals and patients with various malignancies.

	hK6, µg/L					
Patient Group	Number of Samples	Min	Max	Median	95th Percentile	Number of Patients with hK6 > 15µg/L (%)
Normal males	41	3.2	11.4	7.5	11.1	0 (0)
Normal females	40	3.5	13.7	7.0	10.8	0 (0)
Breast cancer[1]	24	1.1	11.9	4.3	9.7	0 (0)
Medullary thyroid carcinoma[2]	29	0	13.9	5.0	11.8	0 (0)
Testicular cancer[3]	78	2.0	32.2	9.3	14.3	1 (2)*
Gastrointestinal cancer[4]	28	2.6	10.6	5.7	9.6	0 (0)
Prostate cancer[5]	40	1.0	16.1	4.1	9.5	1 (2)**
Lung cancer	18	2.6	7.4	5.2	6.7	0 (0)
Ovarian cancer[6]	80	1.0	206	23.0	148	53 (66)

1. With serum CA15.3 levels ≥ 414 KU/L (upper ref. range 35 KU/L).
2. With calcitonin levels ≥ 1,135 ng/L (upper ref. range 100 ng/L).
3. With hCG levels ≥ 69 IU/L (upper ref. range 10 IU/L) or AFP levels ≥ 110 µg/L (upper ref. range 10 µg/L).
4. With CA 19.9 levels ≥ 629 KU/L (upper ref. range 37 KU/L) and CEA levels ≥ 1.000 µg/L (upper ref. range 5 µg/L).
5. With PSA ≥ 324 µg/L (upper ref. range 4 µg/L).
6. With CA 125 ≥ 372 KU/L (upper ref. range 35 KU/L).

Table 3: Descriptive statistics of serum hK6 in non-cancer (healthy), benign disease and ovarian cancer patients.

Variable	Mean ± SE[a]	Range	Percentiles 5	25	50	75	95
Non-Cancer (N=97) hK6 (µg/L)	2.94 ± 0.099	0.89 – 6.58	1.49	2.28	2.90	3.54	4.44
Benign Disease (N = 141) hK6 (µg/L)	3.12 ± 0.074	1.30 – 6.16	1.99	2.50	3.00	3.60	4.88
Pre-Surgical Ovarian Cancer (N=146) hK6 (µg/L)	6.81 ± 0.57	1.30 – 38.00	2.19	3.12	4.40	7.15	25.06
Post-Surgical Ovarian Cancer (N=105) hK6 (µg/L)	3.87 ± 0.25	0.80 – 21.82	1.82	2.66	3.20	4.20	7.72

[a] Standard error

Table 4: Comparison of sensitivity and specificity of serum hK6 concentration at selected cut-off points

Parameter	Cut-Off	Sensitivity (%)	Specificity (%)
Total population (N = 384)	2.20	95	19
hK6 (µg/L))	2.50	90	29
	4.20	54	90
	4.40	50	95
CA125 < 23 KU/L (N = 182)	2.27	95	24
hK6 (µg/L)	2.40	90	28
	4.30	17	90
	4.40	13	95
CA125 23-60 KU/L (N = 65)	2.20	95	10
hK6 (µg/L)	2.40	90	19
	4.00	26	90
	4.20	15	95
CA 125 > 60 KU/L (N = 110)	2.20	95	16
hK6 (µg/L)	2.70	90	43
	4.50	71	90
	5.56	65	95

Table 5: Diagnostic sensitivities for ovarian cancer with CA125 alone, hK6 alone and CA125 + hK6 analysis at 90 and 95% specificity cut-offs for both markers

	Sensitivity at 90% specificity	**Sensitivity at 95% specificity**
All patients with known stage (N=124)		
CA125	60	56
hK6	58	53
CA125+hK6	72	69
Stage I/II patients (N=43)		
CA125	30	26
hK6	26	21
CA125+hK6	42	37

Table 6: Relative risk[a] (RR) of ovarian cancer according to quartiles of serum hK6

	Quartiles (µg/L)			
Parameter	**1** (0.89-2.60) n = 96	**2** (2.61-3.29) n = 96	**3** (3.30-4.27) n = 96	**4** (4.28-38.00) n = 96
hK6 unadjusted [a]				
RR	1.00	1.41	3.12	20.00
95 % confidence intervals		0.71-2.79	1.43-6.85	7.70-48.46
p value		0.32	0.003	< 0.001
hK6 adjusted [b]				
RR	1.00	1.21	2.31	5.33
95% confidence intervals		0.56-2.62	1.05-5.02	2.32-12.24
p value		0.62	0.036	< 0.001

[a] Estimated from unconditional logistic regression models.
[b] Multivariate models were adjusted with the CA125 quartiles.

Table 7: Relationship between hK6 status and other variables in ovarian cancer patients *

Variable	Patients	No. of patients (%) hK6 Negative	hK6 Positive	p Value
Stage				
I	32	27 (84.4)	5 (15.6)	
II	11	8 (72.7)	3 (27.3)	< 0.001[a]
III	73	18 (24.7)	55 (75.3)	
IV	8	3 (37.5)	5 (62.5)	
x	22			
Grade				
G1	39	31 (79.5)	8 (20.5)	
G2	24	7 (29.2)	17 (70.8)	< 0.001[a]
G3	62	19 (30.6)	43 (69.4)	
x	21			
Histotype				
Serous	74	24 (32.4)	50 (67.6)	
Endometrioid	15	10 (66.7)	5 (33.3)	< 0.001[a]
Mucinous	22	20 (90.9)	2 (9.1)	
Others	27	17 (63.0)	10 (37.0)	
x	8			
Residual tumor (cm)				
0	76	52 (68.4)	24 (31.6)	
1-2	17	3 (17.6)	14 (82.4)	< 0.001[a]
> 2	35	6 (17.1)	29 (82.9)	
x	18			
Debulking success[c]				
SO	49	9 (18.4)	40 (81.6)	< 0.001[b]
OD	81	53 (65.4)	28 (34.6)	
x	16			
Response to CTX[d]				
NC/PD	21	4 (19.0)	17 (81.0)	< 0.001[b]
CR/PR	107	61 (57.0)	46 (43.0)	
NE	18			

* hK6 cut-off = 4.4 µg/L (median)
[a] χ^2 test
[b] Fisher's Exact Test
[c] OD, Optimal debulking (0-1 cm); SO, Suboptimal debulking (>1 cm)
[d] CTX, chemotherapy; NC, no change; PD, progressive disease; CR, complete response; PR, partial response; NE, not evaluated
x Status unknown.

Table 8: Univariate and multivariate analysis of serum hK6 in relation to progression-free and overall survival

Variable		Progression-free survival HR[a]	95% CI[b]	p Value	Overall survival HR[a]	95% CI[b]	p Value
		Univariate analysis					
hK6	negative	1.00			1.00		
	positive	4.10	2.28-7.36	< 0.001	3.15	1.36-7.29	0.007
as a continuous variable		1.068	1.041-1.095	< 0.001	1.075	1.038-1.11	< 0.001
CA125	negative[d]	1.00			1.00		
	positive[d]	2.52	1.45-4.38	0.001	2.36	1.03-5.42	0.041
as a continuous variable		1.001	1.000-1.002	< 0.001	1.001	1.000-1.003	0.018
Grading	(ordinal)	2.50	1.71-3.64	< 0.001	2.34	1.53-3.58	< 0.001
Residual tumor	(ordinal)	1.23	1.13-1.34	< 0.001	1.31	1.21-1.41	< 0.001
Histologic type[c]		2.49	1.37-4.54	0.003	4.25	1.44-12.53	< 0.008
		Multivariate analysis					
hK6	negative	1.00			1.00		
	positive	4.86	1.10-21.47	0.036	5.08	1.07-23.69	0.038
as a continuous variable		1.047	1.007-1.089	0.019	1.063	1.007-1.12	0.025
CA125	negative[d]	1.00			1.00		
	positive[d]	2.86	0.69-11.74	0.14	2.17	0.38-63.17	0.38
Stage of disease	(ordinal)	2.54	1.37-4.69	0.003	6.34	2.27-17.7	< 0.001
Grading	(ordinal)	1.63	0.94-2.82	0.078	1.56	0.66-3.68	0.31
Residual tumor	(ordinal)	1.09	0.42-2.26	0.15	1.01	0.80-1.24	0.98
Histologic type[c]		1.08	0.75-1.56	0.65	1.18	0.94-1.31	0.18

[a] Hazard ratio (HR) estimated from Cox proportional hazard regression model
[b] Confidence interval of the estimated HR
[c] Serous vs others
[d] Cut-off = 98 KU/L (95% specificity; 53% sensitivity; 48th percentile).

Table 9. Relationship between hK6 status and other variables in 180 ovarian cancer patients.

Variable	Patients	No. of patients (%) hK6 negative	hK6 positive	P value
Stage				
I	44	38 (86.4)	6 (13.6)	
II	13	8 (61.5)	5 (38.5)	0.034[a]
III	110	72 (65.4)	38 (34.5)	
IV	13	7 (53.8)	6 (46.2)	
Grade				
G1	25	21 (84.0)	4 (16.0)	
G2	27	21 (77.8)	6 (22.2)	0.33[a]
G3	119	84 (70.6)	35 (29.4)	
x	9			
Histotype				
Serous	80	52 (65.0)	28 (35.0)	
Undifferentiated	27	17 (63.0)	10 (37.0)	
Endometrioid	32	27 (46.7)	5 (53.3)	
Mucinous	13	10 (76.9)	3 (13.1)	0.31[b]
Clear cell	14	11 (78.6)	3 (21.4)	
Mullerian	10	8 (80.0)	2 (20.0)	
Others	4	3 (75.0)	1 (25.0)	
Residual tumor (cm)				
0	80	67 (83.2)	13 (16.3)	
1-2	29	16 (55.2)	13 (44.8)	0.002[a]
>2	64	40 (62.5)	24 (37.5)	
x	7			
Menopause				
Pre/peri	50	32 (64.0)	18 (36.0)	0.075[b]
Post	130	99 (76.2)	31 (23.8)	
Response to CTX[c]				
NC/PD	15	11 (73.3)	4 (26.7)	0.99[b]
CR/PR	148	104 (70.3)	44 (29.7)	
NE	17			

[a] χ^2 test.
[b] Fisher's Exact Test
[c] CTX; chemotherapy, NC; no change, PD; progressive disease, CR; complete response, PR; partial response, NE; not evaluated.
x. Status unknown.

Table 10. Univariate and Multivariate Analysis of Prognostic Value of hK6

Variable	Progression-free survival (PFS)			Overall survival (OS)		
	HR[a]	95% CI[b]	P value	HR[a]	95% CI[b]	P value
	Univariate Analysis					
hK6						
Negative	1.00			1.00		
Positive	1.71	1.11-2.64	0.015	1.88	1.09-3.21	0.022
as a continuous variable	1.005	1.002-1.007	0.001	1.004	0.999-1.008	0.074
Stage of disease (ordinal)	2.79	2.07-3.79	<0.001	3.07	2.05-4.61	<0.001
Grading (ordinal)	1.95	1.38-2.75	<0.001	2.07	1.31-3.29	0.002
Residual tumor (ordinal)	1.27	1.20-1.34	<0.001	1.31	1.22-1.41	<0.001
Histologic type[c]	0.83	0.68-1.00	0.055	0.88	0.69-1.13	0.34
Age	1.012	0.99-1.03	0.14	1.015	0.99-1.03	0.15
	Multivariate Analysis					
hK6						
Negative	1.00			1.00		
Positive	1.40	0.84-2.32	0.19	1.08	0.79-1.49	0.62
as a continuous variable	1.002	0.99-1.006	0.22	1.001	0.99-1.004	0.69
Stage of disease (ordinal)	1.57	1.09-2.27	0.014	1.72	1.053-2.82	0.03
Grading (ordinal)	1.31	0.84-2.32	0.18	1.31	0.75-2.25	0.33
Residual tumor (ordinal)	1.14	1.05-1.24	0.001	1.21	1.09-1.34	<0.001
Histologic type[c]	0.95	0.82-1.11	0.57	1.04	0.86-1.26	0.68
Age	1.02	0.99-1.039	0.12	1.02	0.99-1.04	0.21

[a] Hazard ratio (HR) estimated from Cox proportional hazard regression model

[b] Confidence interval of the estimated HR.

[c] Serous vs. others

Table 11. Cox proportional hazard regression analysis for subgroups of patients

Variable	Progression-free survival			Overall survival		
	HR[a]	95% CI[b]	P value	HR[a]	95% CI[b]	P value
Tumor grade I-II						
hK6 univariate	9.25	3.33-25.67	<0.001	5.05	1.63-15.71	0.005
hK6 multivariate[c]	4.29	1.17-15.65	0.027	4.05	1.23-16.6	0.023
Tumor grade III						
hK6 univariate	1.45	0.87-2.39	0.14	1.69	0.91-3.14	0.091
hK6 multivariate[c]	1.03	0.58-1.83	0.91	1.02	0.48-2.13	0.96
Stage I-II						
hK6 univariate	0.90	0.18-4.35	0.89	1.49	0.13-16.53	0.74
hK6 multivariate[d]	1.83	0.17-19.41	0.61	2.23	0.20-25.04	0.51
Stage III-IV						
hK6 univariate	2.04	1.26-3.29	0.004	1.98	1.12-3.47	0.017
hK6 multivariate[d]	1.57	0.93-2.68	0.092	1.33	0.71-2.53	0.37
Optimal debulking success[e]						
hK6 univariate	1.81	0.72-4.55	0.20	2.61	0.70-9.73	0.15
hK6 multivariate[f]	3.75	1.39-10.09	0.019	5.57	1.47-21.04	0.011
Suboptimal debulking success[e]						
hK6 univariate	1.39	0.83-2.32	0.20	1.16	0.64-2.09	0.62
hK6 multivariate[f]	1.27	0.72-2.23	0.40	1.19	0.62-2.27	0.59

[a] Hazard ratio (HR) estimated from Cox proportional hazard regression model

[b] Confidence interval of the estimated HR.

[c] Multivariate models were adjusted for stage of disease, residual tumor, histologic type and age.

[d] Multivariate models were adjusted for tumor grade, residual tumor, histologic type and age.

[e] Optimal debulking (0 - 1 cm residual tumor); suboptimal debulking (> 1 cm residual tumor)

[f] Multivariate models were adjusted for stage of disease, tumor grade, histologic type and age.

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I Claim:

1. A method for detecting hK6 associated with ovarian cancer in a subject comprising:
   (a) taking a sample derived from a subject;
   (b) detecting or identifying in the sample hK6; and
   (c) comparing the detected amount with an amount detected for a standard.
2. A method as claimed in claim 1 which further comprises in step (b) detecting one or more of human stratum corneum chymotryptic enzyme (HSCCE), kallikrein 4, kallikrein 5, kallikrein 8, kallikrein 9, kallikrein 10, CA125, CA15-3, CA19-9, OVX1, lysophosphatidic acid (LPA) or carcinoembryonic antigen (CEA).
3. A method as claimed in claim 1 which further comprises in step (b) detecting one or more of CA 125, kallikrein 9, and kallikrein 10.
4. A method for diagnosing and monitoring ovarian cancer in a subject by quantitating hK6 in a sample from the subject comprising:
   (a) contacting a biological sample from the subject with an antibody specific for hK6 which is directly or indirectly labelled with a detectable substance;
   (b) detecting the detectable substance to quantitate hK6 in the sample;
   (c) comparing the quantitated hK6 to levels for a standard.
5. A method for the diagnosis and monitoring of ovarian cancer comprising
   (a) incubating a biological sample with a first antibody specific for hK6 which is directly or indirectly labeled with a detectable substance, and a second antibody specific for hK6 which is immobilized;
   (b) separating the first antibody from the second antibody to provide a first antibody phase and a second antibody phase;
   (c) detecting the detectable substance in the first or second antibody phase thereby quantitating hK6 in the biological sample; and
   (d) comparing the quantitated hK6 with levels for a standard.
6. A method as claimed in any of the preceding claims wherein the biological sample is serum or tumor tissue extracts.
7. A method as claimed in any of the preceding claims wherein the biological sample is serum.
8. A method as claimed in any of the preceding claims wherein the standard is hK6 levels detected for non-ovarian cancer subjects or subjects with benign disease.
9. A method as claimed in any of the preceding claims wherein detection of an amount of hK6 greater than that of a standard indicates late stage disease, or an increased risk of disease progression and mortality.
10. A method as claimed in claim 5 wherein in step (a) the first and second antibodies are contacted simultaneously or sequentially with the biological sample.
11. A method as claimed in any one of claims 4 to 10 wherein the antibody is a monoclonal antibody, a polyclonal antibody, immunologically active antibody fragments, humanized antibody, an antibody

heavy chain, an antibody light chain, a genetically engineered single chain F_v molecule, or a chimeric antibody.

12. A method as claimed in any one of claims 4 to 10 wherein the detectable substance is alkaline phosphatase.

13. A method as claimed in claim 12 wherein the alkaline phosphatase is detected using a fluorogenic substrate.

14. A method as claimed in any of the preceding claims wherein hK6 is measured using time-resolved fluorescence.

15. A method for imaging a tumor associated with hK6 comprising

(a) incubating the tumor with an agent that binds to hK6 for a sufficient period of time to permit the agent to bind to hK6 associated with the tumor, where the agent carries a label for imaging the tumor;

(b) detecting the presence of the label localized to the tumor.

16. A method as claimed in claim 15 which further comprises in step (a) incubating with one or more of an agent that binds to human stratum corneum chymotryptic enzyme (HSCCE), kallikrein 4, kallikrein 5, kallikrein 8, kallikrein 9, kallikrein 10, CA125, CA15-3, CA19-9, OVX1, lysophosphatidic acid (LPA) or carcinoembryonic antigen (CEA), preferably Ca 125.

17. A method as claimed in claim 16 wherein each agent is labeled so that it can be distinguished in step (b).

18. An in vivo method for imaging ovarian cancer comprising:

(a) injecting a patient with an agent that binds to hK6, the agent carrying a label for imaging the ovarian cancer;

(b) allowing the agent to incubate in vivo and bind to hK6 associated with the ovarian cancer; and

(c) detecting the presence of the label localized to the ovarian cancer.

19. A method as claimed in claim 18 wherein the agent is an antibody which recognizes hK6.

20. A method as claimed in claim 18 or 19 wherein the label is a radiolabel, fluorescent label, nuclear magnetic resonance active label, positron emitting isotope detectable by a positron emission tomography ("PET") scanner, chemiluminescer, or enzymatic marker.

21. A kit for carrying out a method as claimed in any of the previous claims.

22. A kit for carrying out a method as claimed in any of the previous claims comprising an antibody specific for hK6 labeled with an enzyme; and a substrate for the enzyme.

FIGURE 1

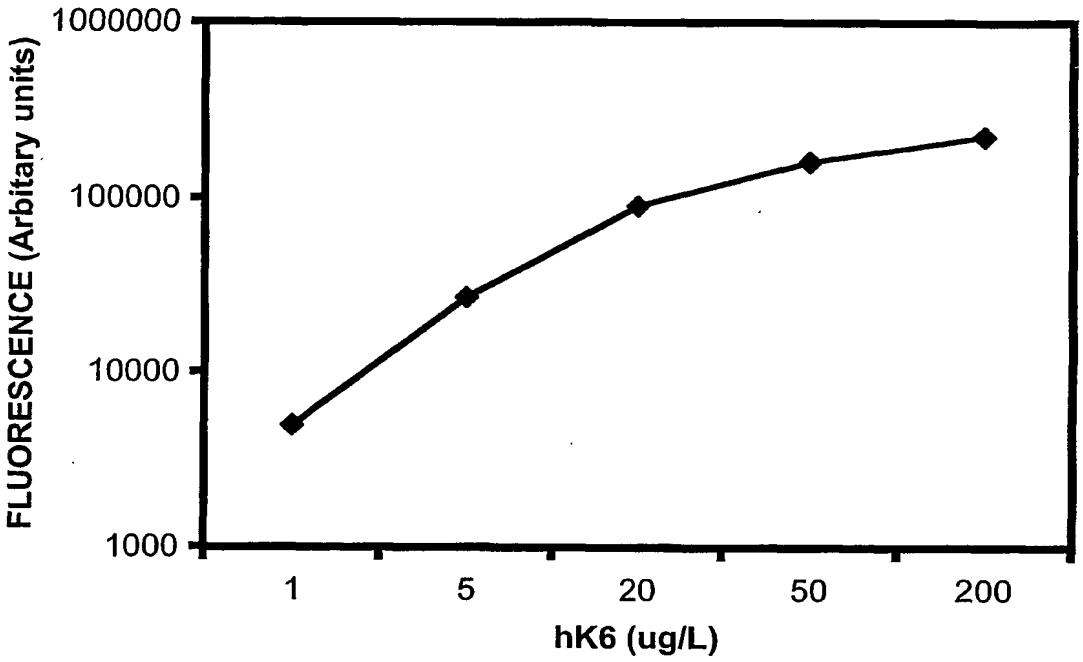

1000000
100000
10000
1000
FLUORESCENCE (Arbitary units)
1
5
20
50
200
hK6 (ug/L)

FIGURE 2

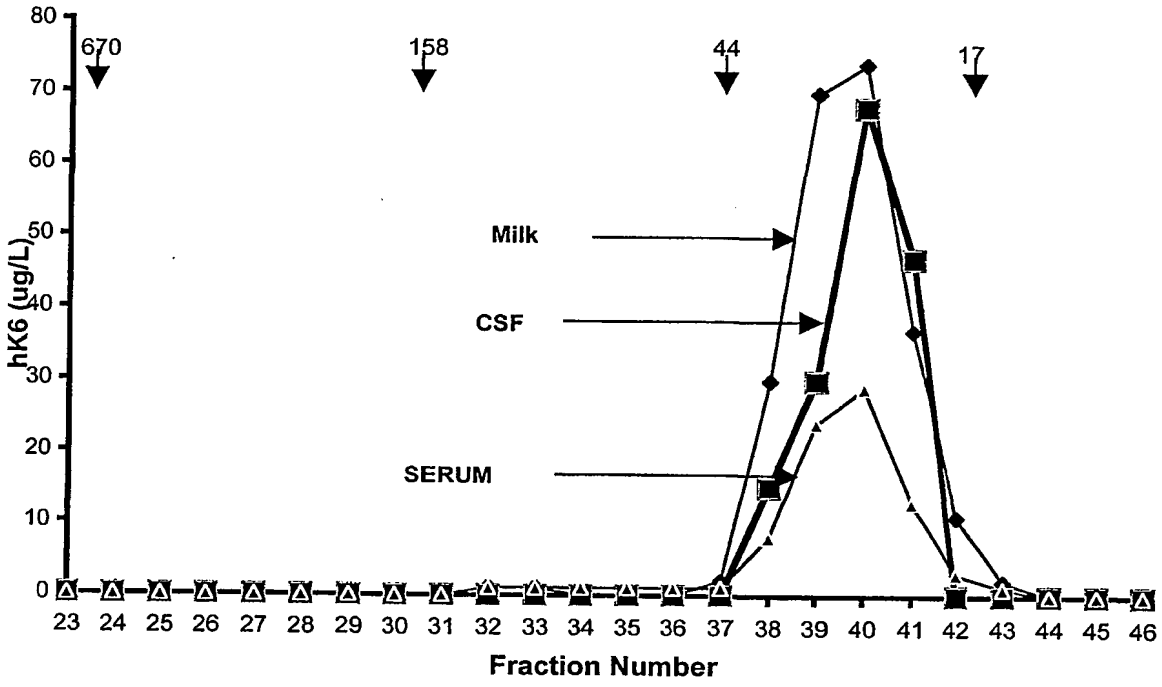

80
70
60
50
40
30
20
10
0
hK6 (ug/L)
670
158
44
17
Milk
CSF
SERUM
23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46
Fraction Number

FIGURE 3

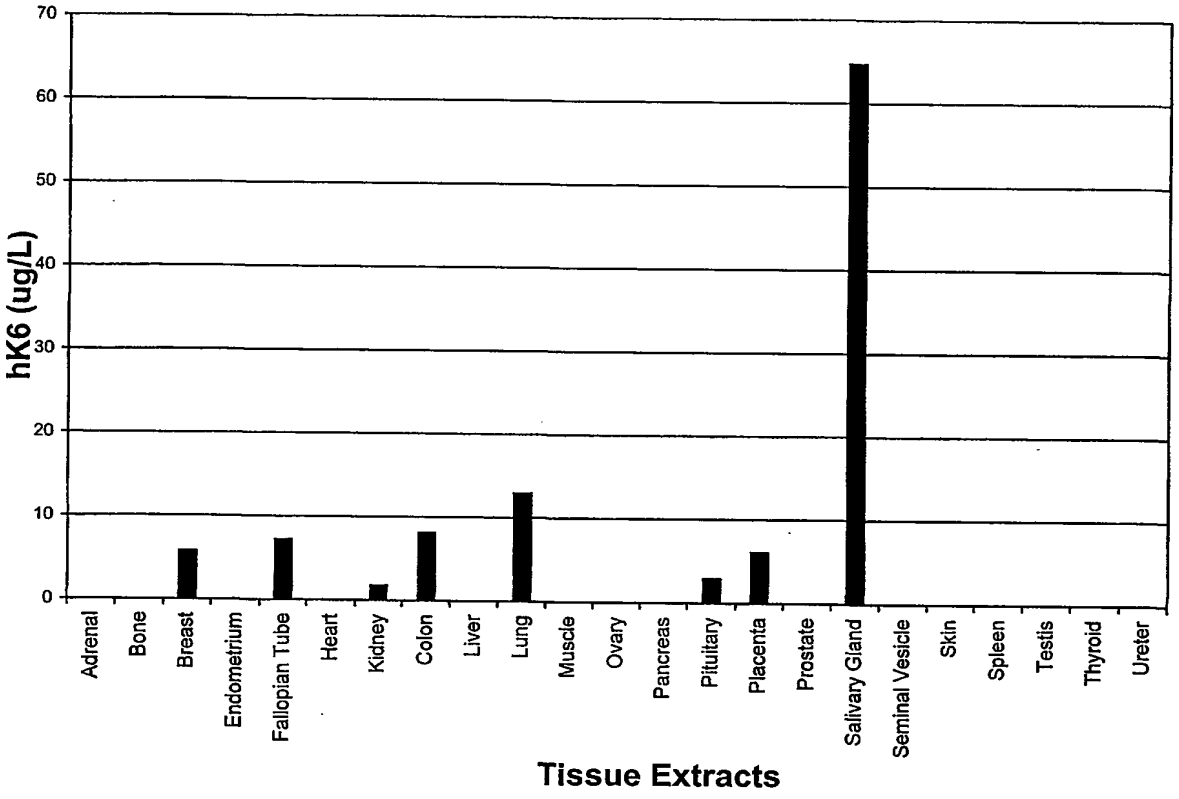

70
60
50
40
30
20
10
0
hK6 (ug/L)
Adrenal
Bone
Breast
Endometrium
Fallopian Tube
Heart
Kidney
Colon
Liver
Lung
Muscle
Ovary
Pancreas
Pituitary
Placenta
Prostate
Salivary Gland
Seminal Vesicle
Skin
Spleen
Testis
Thyroid
Ureter
Tissue Extracts

FIGURE 4

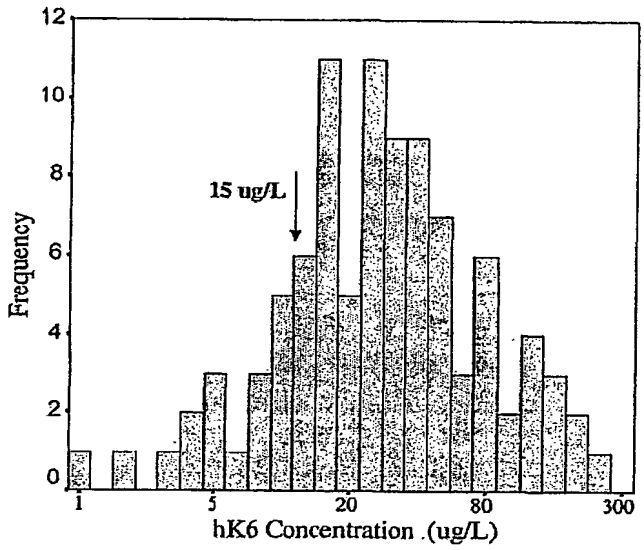

12
10
8
6
4
2
0
Frequency
15 ug/L
1
5
20
80
300
hK6 Concentration .(ug/L)

Figure 5

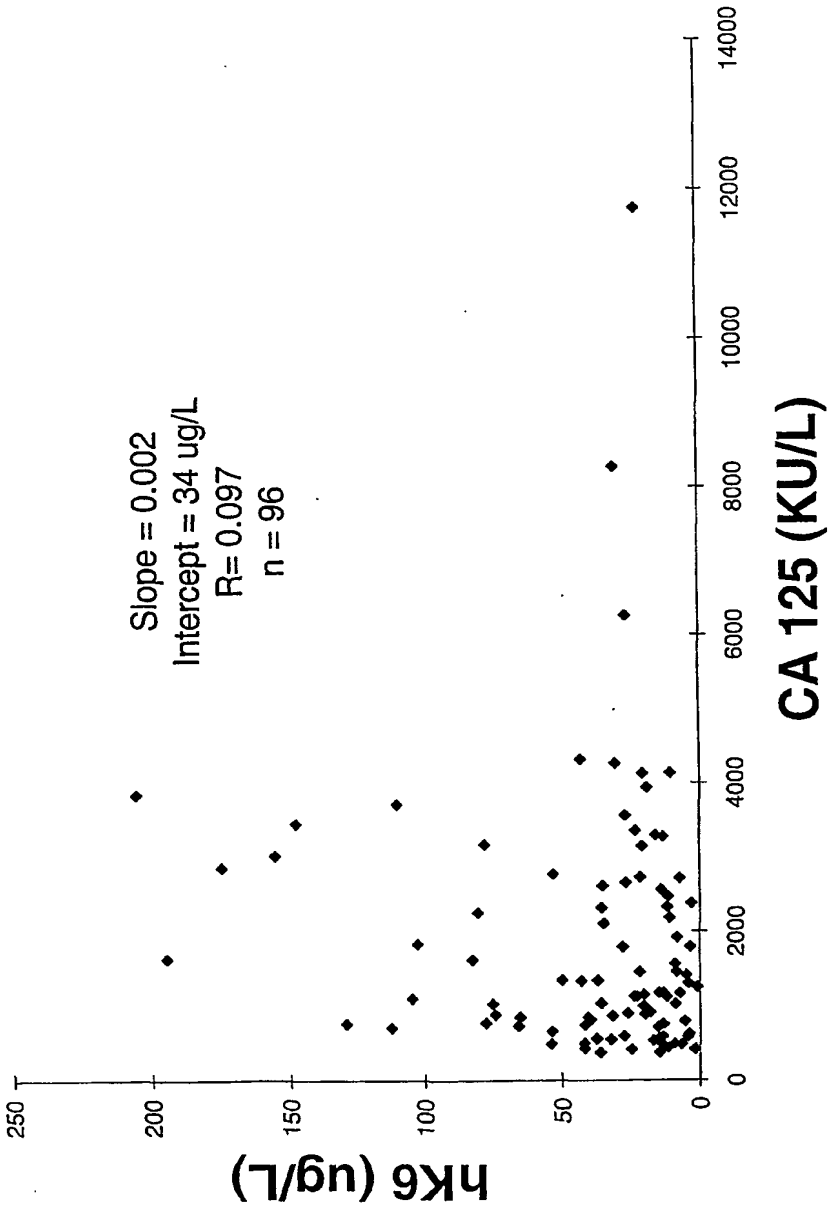

Slope = 0.002
Intercept = 34 ug/L
R= 0.097
n = 96
hK6 (ug/L)
CA 125 (KU/L)
0
50
100
150
200
250
0
2000
4000
6000
8000
10000
12000
14000

FIGURE 6

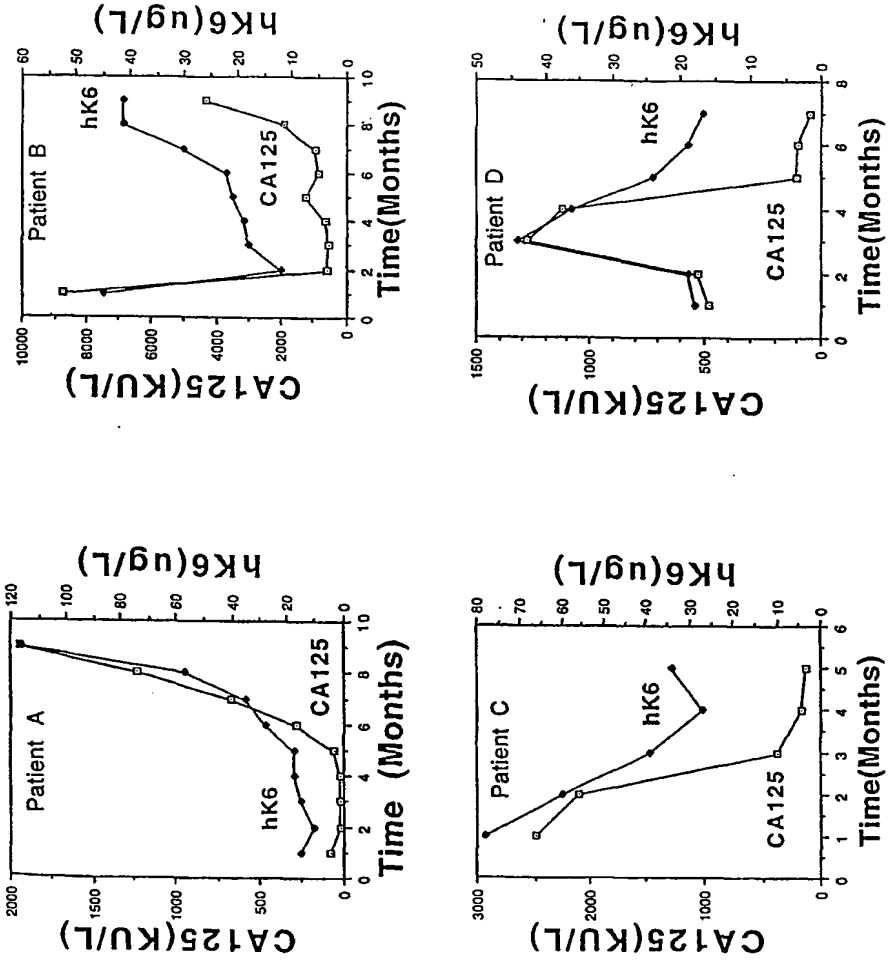

Patient A
Patient B
Patient C
Patient D
hK6
CA125
CA125(KU/L)
hK6(ug/L)
Time (Months)
Time(Months)

Figure 7A

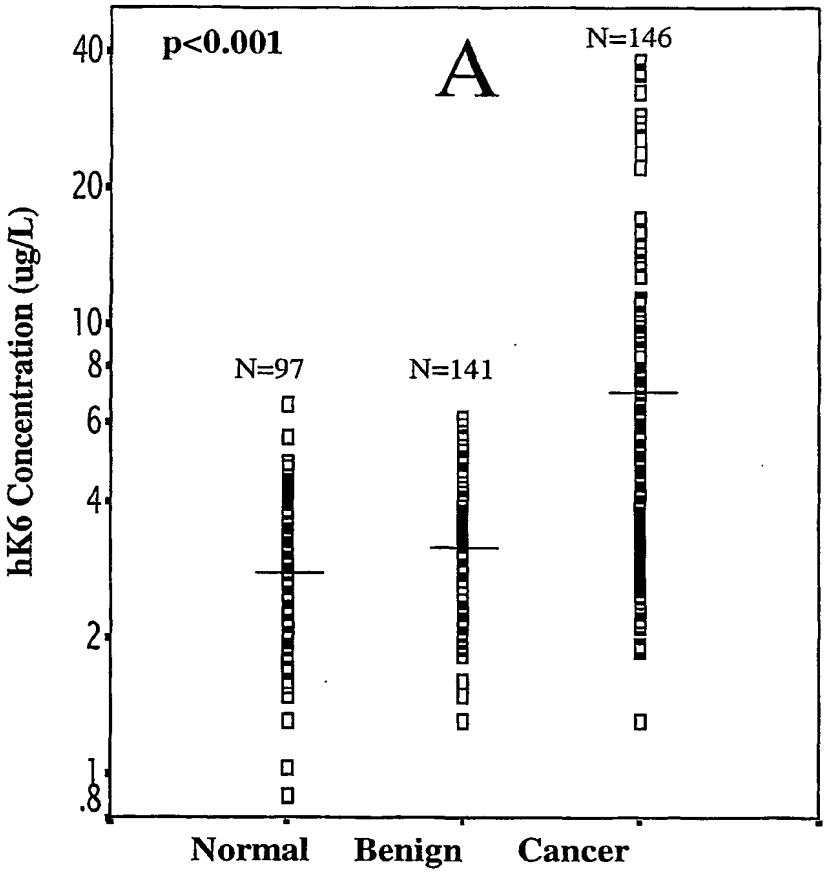

p<0.001
A
N=146
N=97
N=141
hK6 Concentration (ug/L)
40
20
10
8
6
4
2
1
.8
Normal
Benign
Cancer

Figure 7B

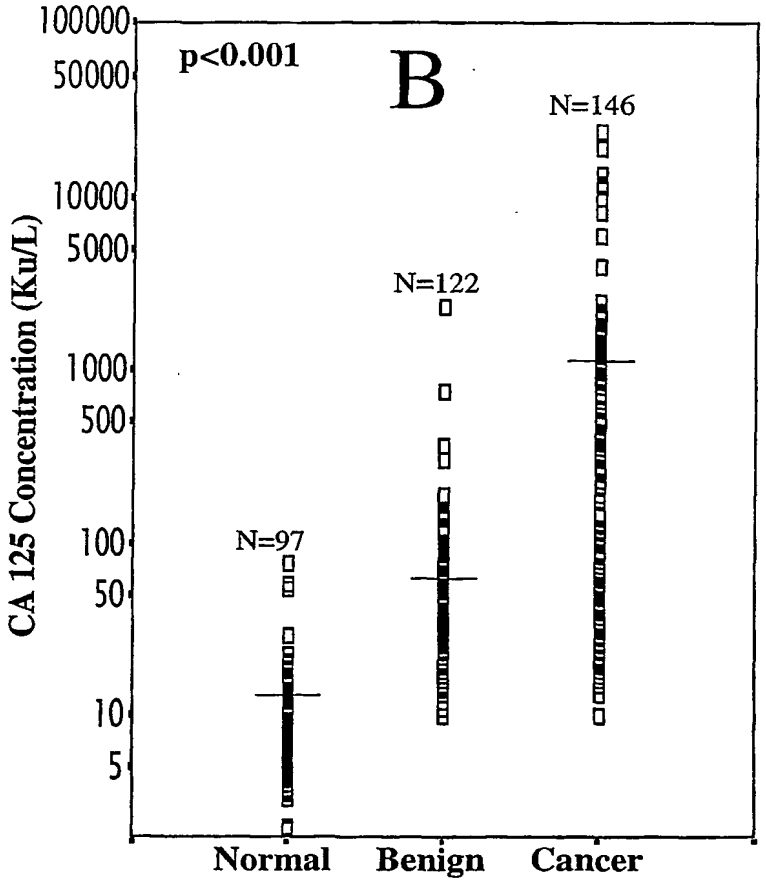

100000
50000
10000
5000
1000
500
100
50
10
5
CA 125 Concentration (Ku/L)
p<0.001
B
N=146
N=122
N=97
Normal
Benign
Cancer

Figure 8

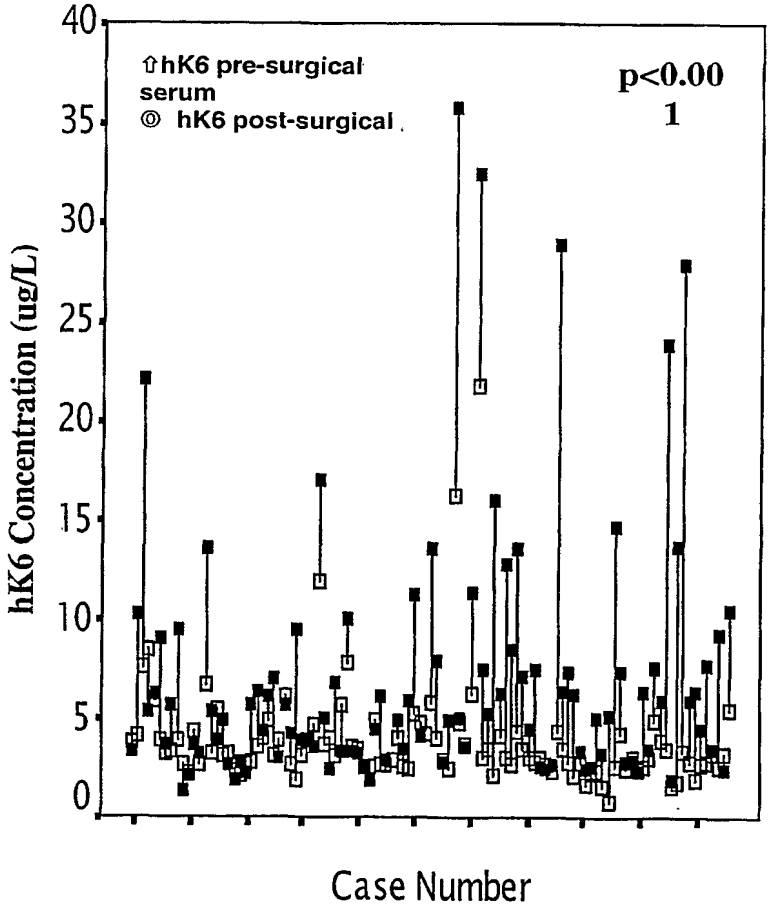

⇧hK6 pre-surgical serum
◎ hK6 post-surgical
p<0.00 1
hK6 Concentration (ug/L)
40
35
30
25
20
15
10
5
0
Case Number

FIGURE 9

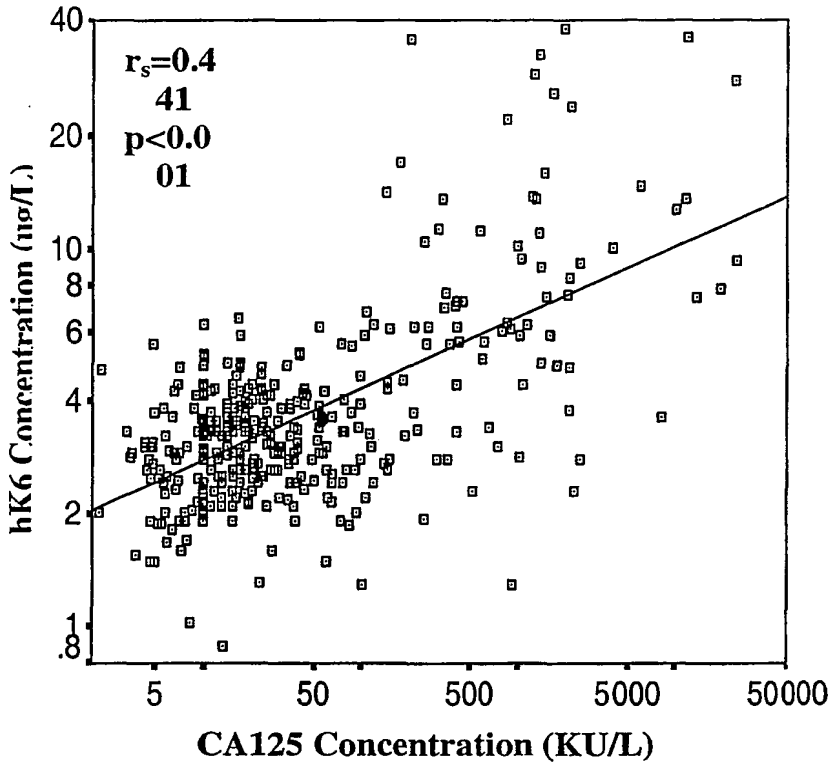

r_s=0.441
p<0.001
hK6 Concentration (ng/L)
40
20
10
8
6
4
2
1
.8
5
50
500
5000
50000
CA125 Concentration (KU/L)

FIGURE 10

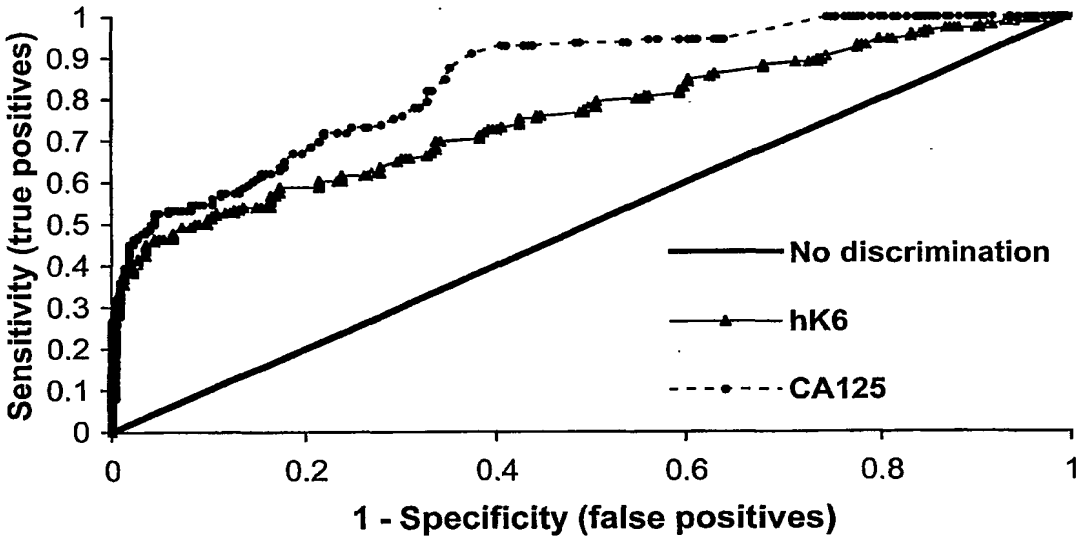

Sensitivity (true positives)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1
1 - Specificity (false positives)
No discrimination
hK6
CA125

Figure 11A

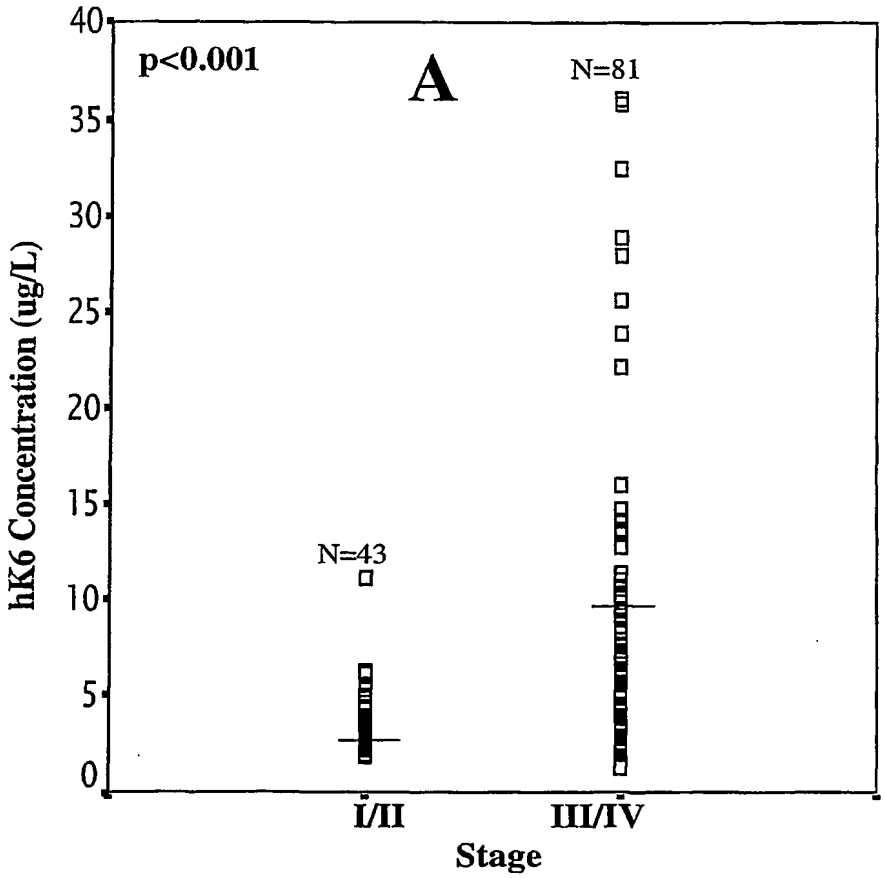

p<0.001
A
N=81
N=43
hK6 Concentration (ug/L)
40
35
30
25
20
15
10
5
0
I/II
III/IV
Stage

Figure 11B

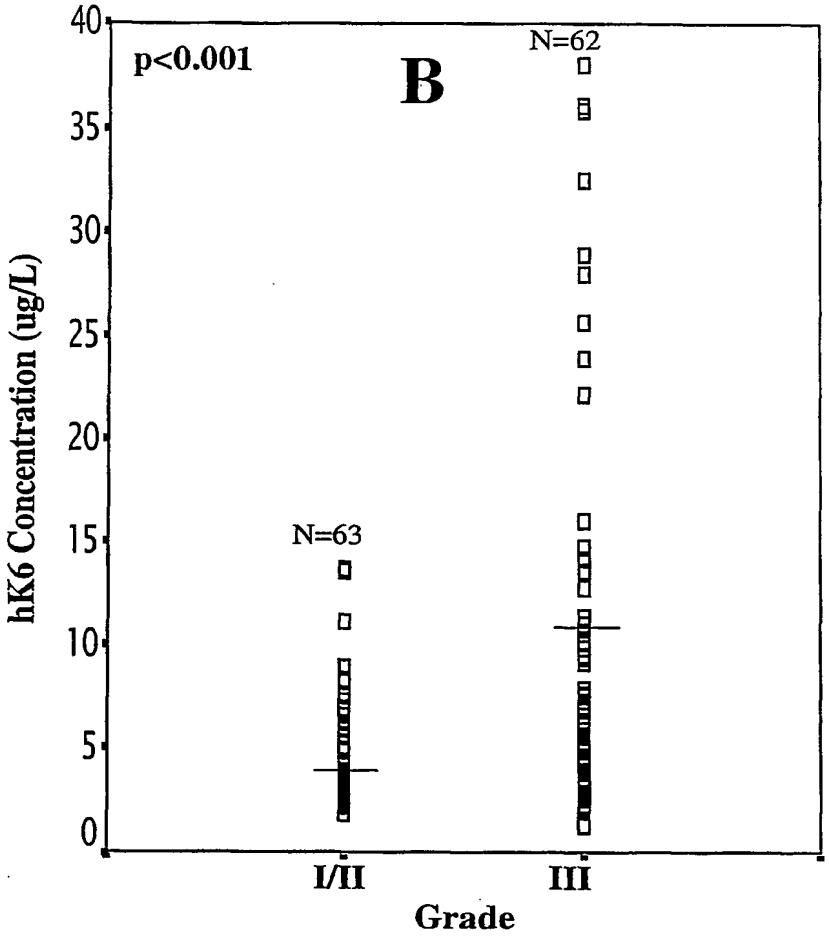

p<0.001
B
N=62
N=63
hK6 Concentration (ug/L)
40
35
30
25
20
15
10
5
0
I/II
III
Grade

Figure 12A

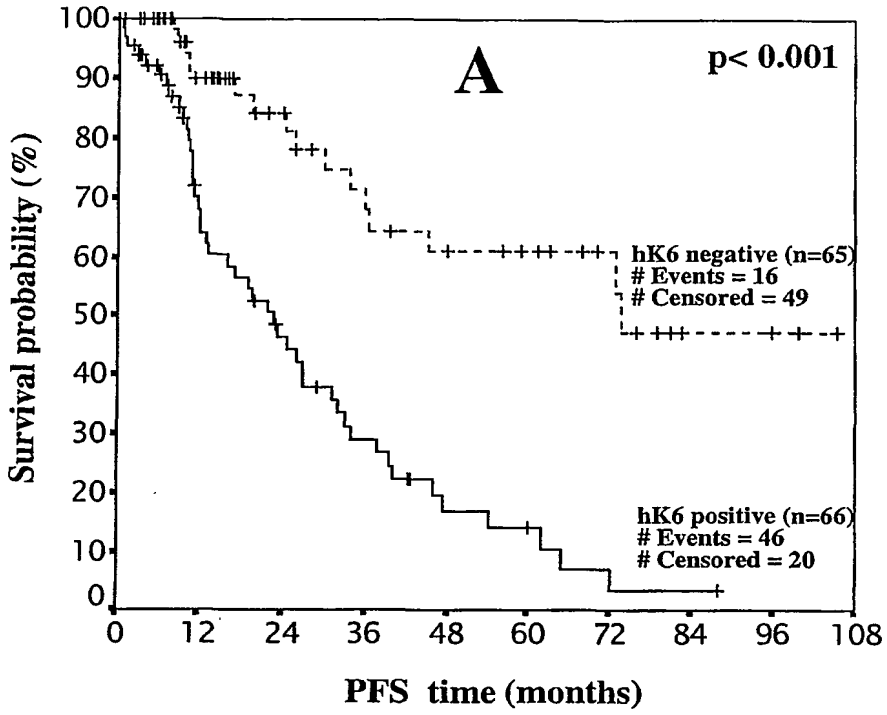

A
p< 0.001
Survival probability (%)
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
60
72
84
96
108
PFS time (months)
hK6 negative (n=65)
Events = 16
Censored = 49
hK6 positive (n=66)
Events = 46
Censored = 20

Figure 12B

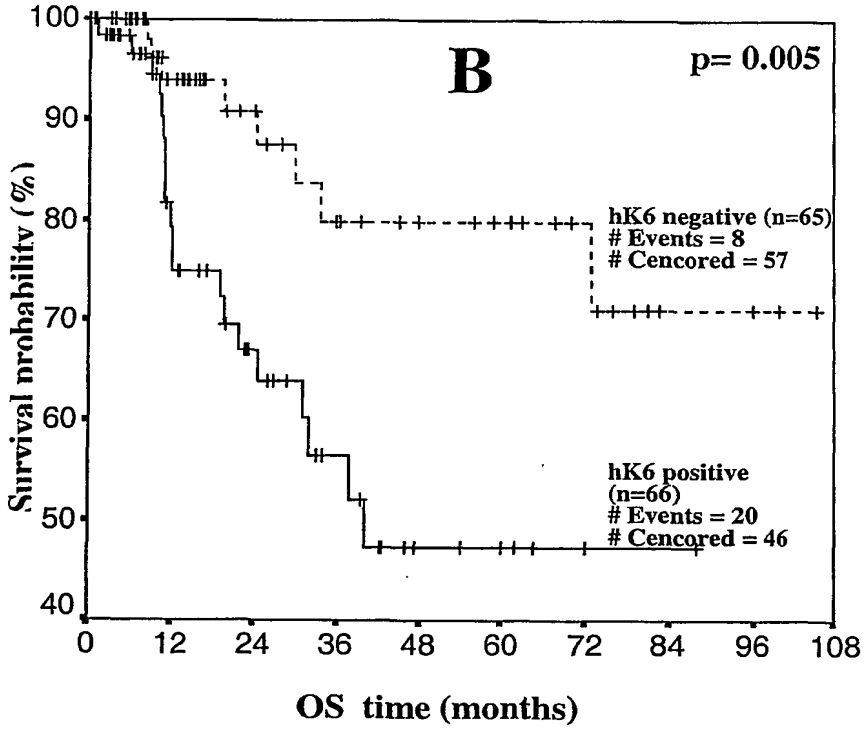

B
p= 0.005
hK6 negative (n=65)
Events = 8
Cencored = 57
hK6 positive
(n=66)
Events = 20
Cencored = 46
Survival probability (%)
100
90
80
70
60
50
40
0
12
24
36
48
60
72
84
96
108
OS time (months)

Figure 13A

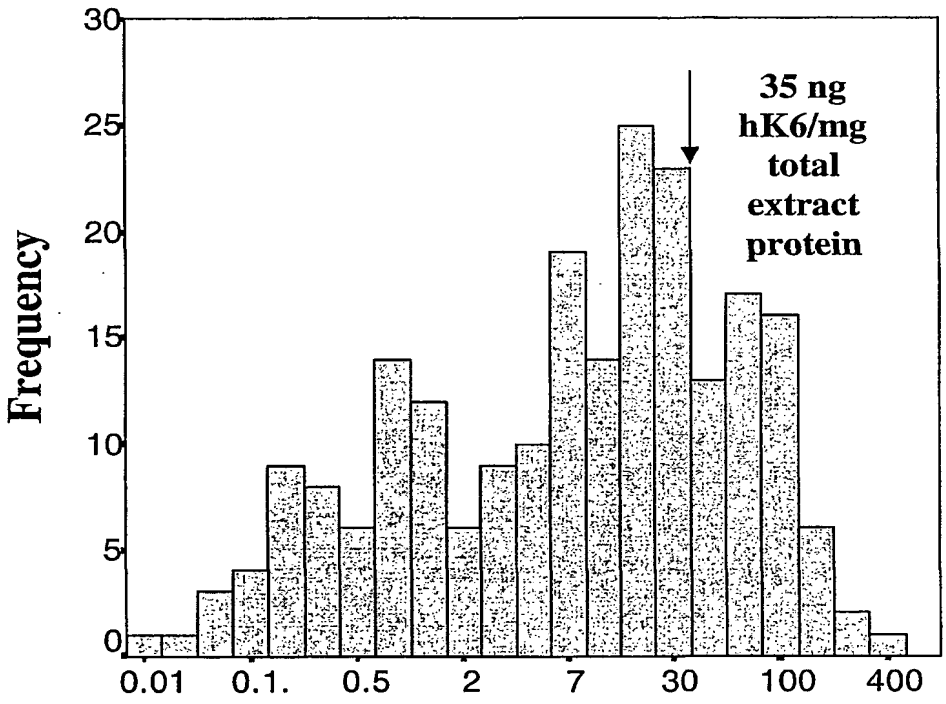

30
25
20
15
10
5
0
Frequency
35 ng hK6/mg total extract protein
0.01
0.1.
0.5
2
7
30
100
400
hK6 (ng/mg extract total protein)

Figure 13B

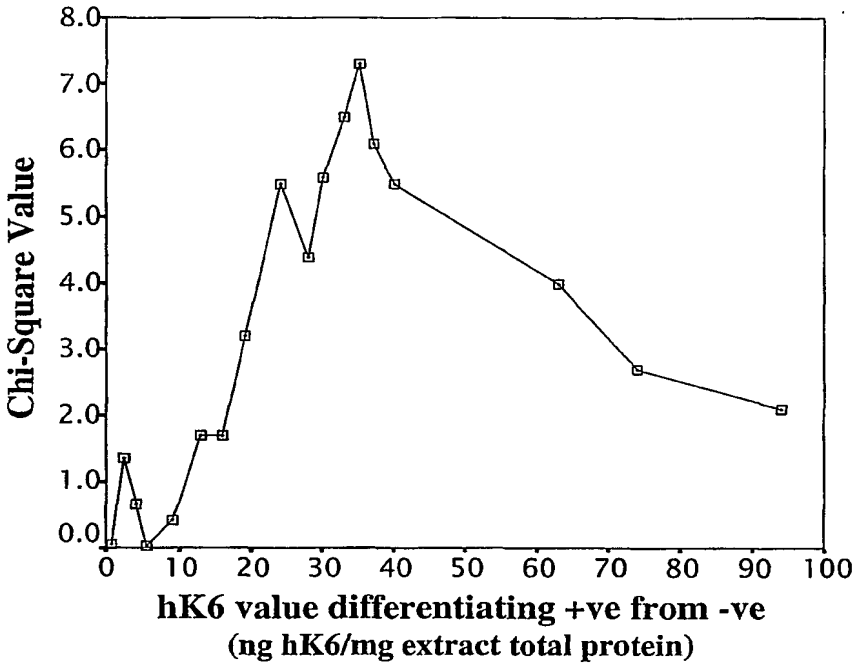

Chi-Square Value
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
40
50
60
70
80
90
100
hK6 value differentiating +ve from -ve
(ng hK6/mg extract total protein)

Figure 14

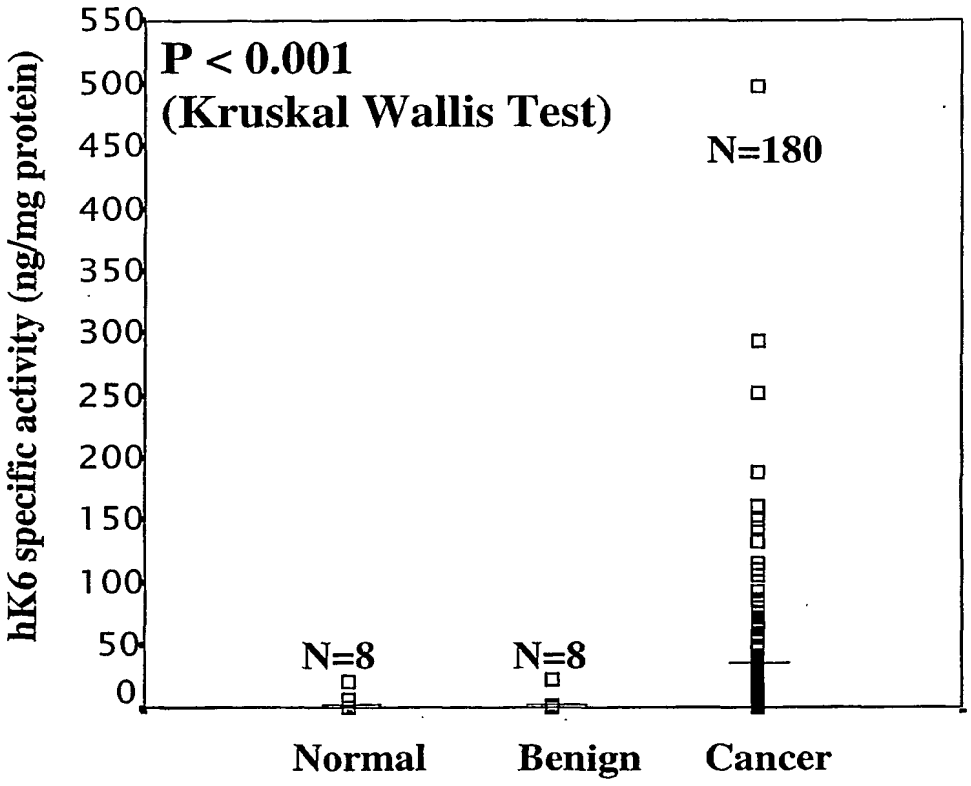
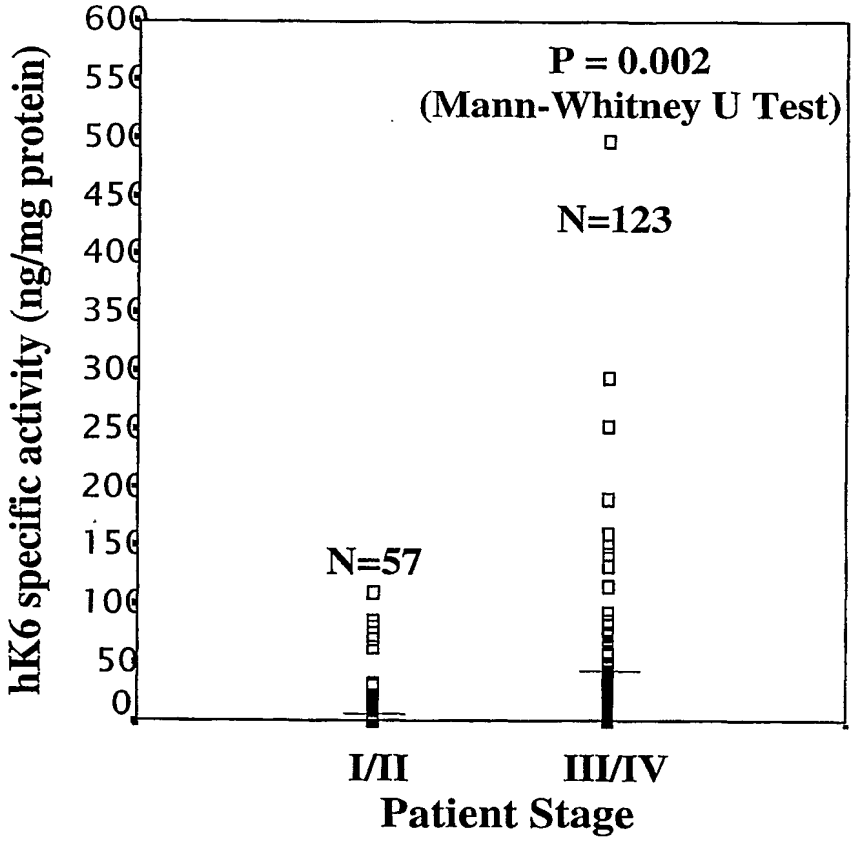

hK6 specific activity (ng/mg protein)
550
500
450
400
350
300
250
200
150
100
50
0
P < 0.001
(Kruskal Wallis Test)
N=180
N=8
N=8
Normal
Benign
Cancer

Figure 15


hK6 specific activity (ng/mg protein)
600
550
500
450
400
350
300
250
200
150
100
50
0
P = 0.002
(Mann-Whitney U Test)
N=123
N=57
I/II
III/IV
Patient Stage

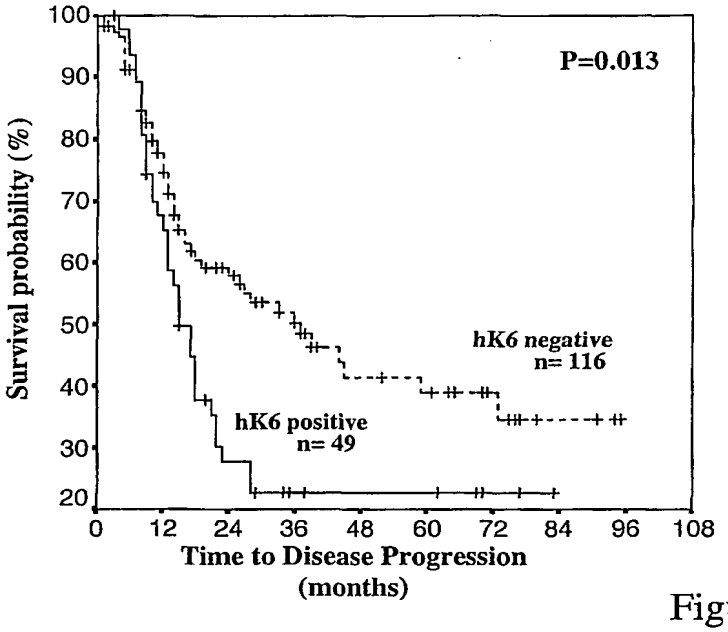
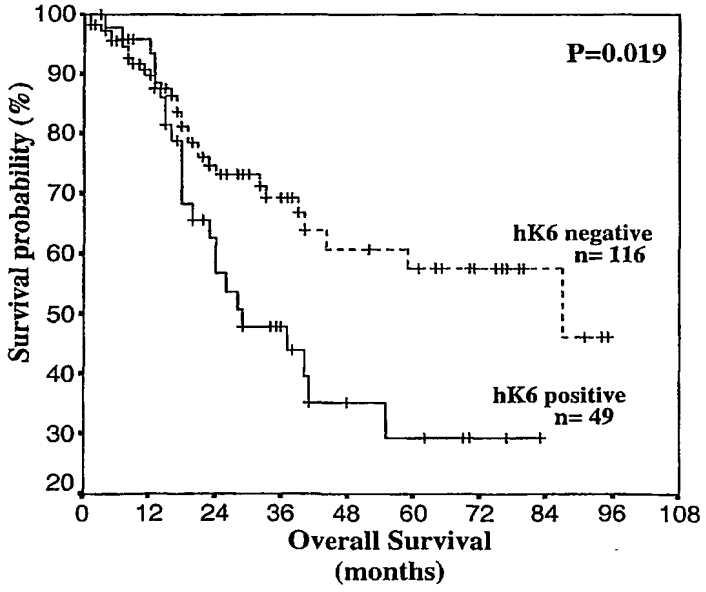

P=0.013
hK6 negative
n= 116
hK6 positive
n= 49
Survival probability (%)
100
90
80
70
60
50
40
30
20
0
12
24
36
48
60
72
84
96
108
Time to Disease Progression
(months)

Figure 16


P=0.019
hK6 negative
n= 116
hK6 positive
n= 49
Survival probability (%)
100
90
80
70
60
50
40
30
20
0
12
24
36
48
60
72
84
96
108
Overall Survival
(months)

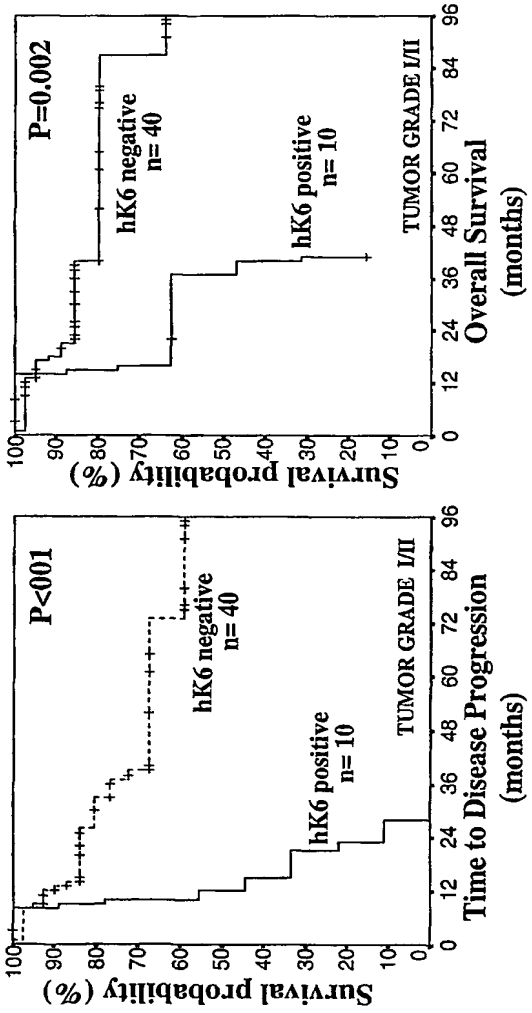

P<001
hK6 negative n= 40
hK6 positive n= 10
TUMOR GRADE I/II
Survival probability (%)
Time to Disease Progression (months)
P=0.002
hK6 negative n= 40
hK6 positive n= 10
TUMOR GRADE I/II
Survival probability (%)
Overall Survival (months)

Figure 17

Figure 18